DOCTOR OF PHILOSOPHY

Investigation into the effects of Artemisinin in myocardial ischaemia reperfusion injury

Aminu Babba, Maryam

Award date:
2015

Awarding institution:
Coventry University

Investigation into the effects of Artemisinin in myocardial ischaemia reperfusion injury.

A thesis submitted in partial fulfilment of the requirements for the degree of Doctor of Philosophy in Cardiovascular Physiology and Pharmacology.

Coventry University

July 2015

Maryam Aminu Babba

Supervisory Team: Dr Afthab Hussain, Professor HL Maddock and Dr Omar Janneh

ACKNOWLEDGEMENT

I would like to express my sincere gratitude to my Director of studies, Dr Afthab Hussain, my supervisor and employer Professor Helen Maddock and Dr Omar Janneh for their continuous support during my Ph.D, their motivation and guidance. They did not just coach me but they mentored me throughout this period. They took time out from their busy scheduled/while on annual leave to read my thesis. I sincerely feel indebted to you Dr Hussain and Prof Maddock.

I would also like to thank you again and Coventry University for funding my project. Besides my supervisory team, I would also like to thank my colleagues and friends in the lab (this is in alpahabetical order no preference whatsoever): Mark Bodycote, Shabana Cassamabai, Oana Chiuzbian, Samantha Cooper, Bethan Grist, Mayel Gharanei, Kate Harvey, Cameron Hill, Jawad Khan, Aaron Nagra, Lidia Pisula, Hardip Sandhu, and Andre Varciana for their support, teaching me some of the techniques, the stimulating discussions, the pep talks, taking care of the animals and above all their friendship. You all made the long hours in the lab a lot more fun.

It is with immense gratitude that I acknowledge the support and help of my family: my father, my mother, my husband (sorry Shams had to mention Abba first ;)), my siblings, my daughter, and the friends that have become family along the way. I cannot begin to list you all but by Allah I owe you all my eternal gratitude. Thank you for supporting me throughout my Ph.D and through life too. For your encouraging words, insightful comments, for not getting mad when I took my laptop on family outings and for your editing assistance and for reading my thesis. Without you all, Lord knows where I will be. I love you and I thank you.

Collectively, you have all made a difference in my life. I have grown as a researcher and as a person. Thank you for your directions, technical support, being mentors and friends. The times when I felt like giving up, your persistence, understanding and kindness saw me through. I doubt that I will ever be able to convey my appreciation fully, but I am sincerely grateful. I want to thank you all over again and acknowledge you all individually.

Thank you,

Maryam Aamatullah Babba

ABSTRACT

Artemisinin is herbal drug with a wide range of biological and physiological function. It is currently administered in the treatment against uncomplicated *F.Palcifarum* infections. It has also been shown to be cytotoxic against a variety of cancer cells. Despite the promise of many anti cancer drugs, drug induced cardiotoxicity has constantly threatened drug applicability especially in patients with co-morbities. Artemisinin has been shown to be cardioprotective, although the intracellular pathways remain to be elucidated.

In this study, isolated perfused rat hearts were subjected to 35 minutes of ischaemia and 120 minutes reperfusion or primary cardiac myocytes subjected to 120 minutes hypoxia and 120 minutes reoxygenation where artemisinin (4.3µM) was administered in presence and absence of the PI3K inhibitor (wortmannin) (0.1µM), p70S6K inhibitor (rapamycin) (0.1µM), non selective nitric oxide synthase inhibitor (L-NAME) (100µM) and inducible nitric oxide synthase inhibitor (aminoguanidine) (100µM). At the end of the experiment, hearts underwent infarct size to risk ratio assessment via tri-phenyltetrazolium chloride staining or western blot analysis for p-Akt and p70S6K. Cardiac myocytes were assessed for either MTT analysis, cleaved-caspase 3 or for eNOS/iNOS or p-BAD activity using flow cytometry.

In isolated hearts, artemisinin (0.1µM-100µM) showed a significant dose dependent decrease in infarct size ($P<0.01$-0.001 vs. I/R control). It was also shown to significantly improve cellular viability (66.5±6.3% vs. 29.3±6.1% in H/R, $P<0.01$) and decrease the levels of cleaved caspase-3 compared to the H/R control group (17.1±2.0% vs. 26.8±2.0% in H/R, $P<0.001$). Artemisinin was shown to confer protection via the activation of the PI3K-Akt-p70S6k cell survival pathway and presented an upregulation in p-eNOS and iNOS expression. Furthermore, co-administering artemisinin with doxorubicin showed artemisinin reverses I/R or H/R injury as well as doxorubicin-induced injury via the nitric oxide signalling pathway. Additionally, in HL-60 cells, the co-administration doubled artemisinins cytotoxicity while also implicating the nitric oxide pathway. This is the first study to shows that artemisinin ameliorates doxorubicin mediated cardiac injury whilst enhancing its cytotoxicity in HL-60 in a nitric oxide dependent manner. This study concluded that artemisinin was both anti apoptotic and protective against myocardial I/R injury via the PI3K-Akt-BAD/P70S6K and via the nitric oxide cell survival pathway as well as pro-apoptotic against HL-60 in a nitric oxide dependent manner.

Table of Contents

LIST OF FIGURES

Figure 19. Infarct to risk ratios in the isolated rat hearts subjected to 20 minutes of stabilisation followed by 155 minutes perfusion with KH buffer in the presence/absence of artemisinin (4.3µM). Results were expressed as Mean±SEM (*P<0.05 vs. normoxic control) (n=6).

Figure 20a: Area at risk expresses as a percentage of the left ventricular volume. Hearts were subjected to 20 minutes stabilisation, 35 minutes ischaemia and 120 minutes reperfusion in the presence/absence of artemisinin (0-100µM). Results were expressed as Mean±SEM.

Figure 20b. The effects of artemisinin (0-100µM) on infarct size to risk ratios in isolated perfused rat heart model of I/R. Hearts were subjected to 20 minutes stabilisation, 35 minutes ischaemia and 120 minutes reperfusion in the presence/absence of artemisinin (0-100µM). Results were expressed as Mean±SEM (**P<0.01 vs. I/R control, ***P<0.001 vs. I/R control) (n=4-8) (Artemisinin=Art).

Figure 20c. Dose response curve used to calculate the EC_{20}, EC_{50} and EC_{80} values as 0.042µM, 0.43µM and 4.3µM using values from Figure 10a. Graphs shows the effect of increasing artemisinin concentration (0-100µM) on infarct size to risk ratios in isolated perfused rat heart model of I/R. Hearts were subjected to 20 minutes stabilisation, 35 minutes ischaemia and 120 minutes reperfusion in the presence/absence of artemisinin (0-100µM)(n=4-8).

Figure 21. The effects of artemisinin ($EC_{20:}$ 0.042µM, $EC_{50:}$ 0.43µM or EC_{80}: 4.3µM) on LVDP in isolated perfused hearts subjected to 20 minutes of stabilisation, 35 minutes and ischaemia and 120 minutes reperfusion in the presence/absence of artemisinin ($EC_{20:}$ 0.042µM, $EC_{50:}$ 0.43µM or EC_{80}: 4.3µM). Data expressed as a % of mean stabilisation±SEM. artemisinin (4.3µM) *P<0.05 vs. I/R control, artemisinin (4.3µM) **P<0.01 vs. I/R control (n= 4-8).

Figure 22. The effects of artemisinin ($EC_{20:}$ 0.042µM, $EC_{50:}$ 0.43µM or EC_{80}: 4.3µM) on H/R in isolated perfused hearts subjected to 20 minutes of stabilisation, 35 minutes and ischaemia and 120 minutes reperfusion in the presence/absence of artemisinin ($EC_{20:}$ 0.042µM, $EC_{50:}$ 0.43µM or EC_{80}: 4.3µM). Data expressed as a % of mean stabilisation±SEM. (n= 4-8).

Figure 23. The effects of artemisinin ($EC_{20:}$ 0.042µM, $EC_{50:}$ 0.43µM or EC_{80}: 4.3µM) on CF in isolated perfused hearts subjected to 20 minutes of stabilisation, 35 minutes and ischaemia and 120 minutes reperfusion in the presence/absence of artemisinin ($EC_{20:}$ 0.042µM, $EC_{50:}$ 0.43µM or EC_{80}: 4.3µM). Data expressed as a % of mean stabilisation±SEM.*P<0.05 vs I/R control) (n= 4-8).

Figure 24. The effects of artemisinin (4.3µM) on infarct to risk ratios in isolated perfused rat heart model subjected to 20 minutes stabilisation, 35 minutes ischaemia and 120 minutes reperfusion in the presence/absence of artemisinin (4.3µM) which was administered throughout reperfusion. Results were expressed as Mean±SEM (***P<0.01 vs. I/R control) (n=6).

Figure 25. Mean fold changes in the differential expression of miRNA-1 in hearts subjected to 20 minutes stabilisation, 35 minutes ischaemia and 120 minutes reperfusion in the presence/absence of artemisinin (4.3µM) where administered throughout reperfusion. Results were expressed as Mean±SEM. (*P<0.05 vs I/R Control) (n=3-4).

Figure 26. Mean fold changes in the differential expression of miRNA-27a in artemisinin (4.3µM) treated hearts compared to I/R control. Hearts were subjected to 20 minutes stabilisation, 35 minutes ischaemia and 120 minutes reperfusion in the presence/absence of artemisinin (4.3µM) administered throughout reperfusion. Results were expressed as Mean±SEM. (n=3-4).

Figure 27. Mean fold changes in the differential expression of miRNA-133a in artemisinin (4.3µM) treated hearts compared to I/R control. Hearts were subjected to 20 minutes stabilisation, 35 minutes ischaemia and 120 minutes reperfusion in the presence/absence of artemisinin (4.3µM) which was administered throughout reperfusion. Results were expressed as Mean±SEM (**P<0.01 vs. IR control) (n=3-4).

Figure 28. Mean fold changes in the differential expression of miRNA-133b in artemisinin (4.3µM) compared to control. Hearts were subjected to 20 minutes stabilisation, 35 minutes ischaemia and 120 minutes reperfusion in the presence/absence of artemisinin (4.3µM) which was administered throughout reperfusion. Results were expressed as Mean±SEM (n=3-4).

Figure 29. Mean fold changes in the differential expression of miRNA-155 in artemisinin (4.3µM) treated hearts compared to I/R hearts. Hearts were subjected to 20 minutes stabilisation, 35 minutes ischaemia and 120 minutes reperfusion in the presence/absence of artemisinin (4.3µM) which was administered throughout reperfusion. Results were expressed as Mean±SEM (n=3-4).

Figure 30. % Effect of administration of artemisinin (Art) (4.3µM) on cleaved casapase-3 levels as analysed by Flow cytometry. Results were expressed as Mean±SEM **(*****P<0.001 vs Normoxia, *P<0.05 vs H/R) (n=4-6).

Figure 31. % change in MTT reductase activity compared with control in isolated rat ventricular myocytes following H/R. Cardiomyocytes were subjected to 120 minutes hypoxia followed by 120 minutes of reoxygenation in the presence/absence of artemisinin (4.3µM). Results are expressed as Mean±SEM (***P<0.001 vs. Normoxic control and *P<0.01 vs. H/R) (n=6).

Figure 32a. Dose response curve showing the effect of MTT cytotoxicity analysis on HL-60 cells in response to treatment with increasing concentrations of artemisinin (1-1000µM). HL-60 cells were incubated with artemisinin (1-1000µM) for 24 hours. Experiment was terminated by adding MTT solution and cells were further incubated for 2 hours until termination with lysis buffer. Plate readings were taken at 492nm and results analysed using the 4-parametric logistic analysis, Graphit software. Image of HL-60 control cells and artemisinin (0.4mM) cells shown below (Figure 32b).

Figure 32b. HL-60 cells control vs artemisinin treated cells. Image shows HL-60 cells to the right incubated with artemisinin (0.4mM) for picture purposes and to the left is untreated HL-60 control.

Figure 32c. % change in MTT reductase activity compared to control in MTT cytotoxicity analysis of HL-60 cells in response to treatment with artemisinin (0.4mM). HL-60 cells were incubated with artemisinin (0.4mM) for 24 hours. Experiment was terminated by adding MTT solution and cells were further incubated for 2 hours until termination with lysis buffer. Plate readings were taken at 492nm and results analysed using the 4-parametric logistic analysis, Graphit software.

Figure 33. Treatment protocol for infarct size assessment studies and ventricular myocytes studies respectively.

Figure 34. Illustration of a typical western blot set up used to quantify protein.

Figure 35. Assessment of PI3K-Akt cell survival pathway in Artemisinin mediated cardioprotection in isolated perfused heart model subjected to 35 minutes ischaemia followed

by 120 minutes reperfusion. Artemisinin (Art) (4.3μM) (EC20) was administered at reperfusion in the presence and absence of wortmannin (Wort) (PI3K inhibitor) (0.1μM) or rapamycin (Rapa) (mTOR inhibitor) (0.1μM). Wortmannin (0.1μM) or rapamycin (0.1μM) was also administered alone at reperfusion and subjected to same experimental protocol. Results are shown as Mean±SEM. (***P<0.001 vs IR, **P<0.01 vs Art and *P<0.05 vs Art) (n=6-8).

Figure 36. % change in MTT reductase activity compared with control in isolated rat ventricular myocytes following hypoxia/reoxygenation protocol. For all drug treated groups except .Artemisinin (Art) (4.3μM) was administered at the start of reoxygenation with/without co-administering the inhibitors wortmannin (Wort) (PI3K inhibitor) (0.1μM) or rapamycin (Rapa) (mTOR inhibitor) (0.1μM). The inhibitors, wortmannin (0.1μM) or rapamycin (0.1μM) were also administered alone at reoxygenation and subjected to same experimental protocol. Values are mean ± SEM (***P<0.001 vs Norm, *P<0.05 vs Norm, **P<0.01 vs H/R, #P<0.05 vs Art and ##P<0.01 vs Art) (n=6).

Figure 37. % increase in Cleaved Caspase-3 activity comparedn to normoxia showing the effect administering Artemisinin (Art) (4.3μM) at the start of reoxygenation with/without co-administering inhibitors wortmannin (Wort) (PI3K inhibitor) (0.1μM) and rapamycin (Rapa) (mTOR inhibitor) (0.1μM) . The inhibitors, wortmannin (0.1μM) or rapamycin (0.1μM) were also administered alone at reoxygenation and subjected to same experimental protocol. Values are Mean±SEM (***P<0.001 vs Norm, *P<0.05 vs H/R, # P<0.05 vs Art and ##P<0.01 vs Art) (n=4-6).

Figure 38a. A representative blot of p-Akt and Total Akt from isolated rat heart tissue in response to the treatment with artemisinin (Art) (4.3μM), a combination of Art (4.3μM) and wortmannin (Wort) (0.1μM) (ie Art+Wort) and Wort (0.1μM) alone in the ischaemic reperfusion model.

Figure 38b: Western blot demonstrating Phospho-Akt $_{(Ser473)}$ expression with artemisinin (Art) 4.3μM and wortmannin (Wort) (PI3K inhibitor) (0.1μM) which abolishes expression induced by artemisinin. Wortmannin (0.1μM) was also administered alone at reperfusion and subjected to same experimental protocol as the artemisinin treatment. (**P<0.01 vs IR and ##P<0.05 vs Art)(n=4).

Figure 39a: Above is the representative blot of of p-p70S6K and T-p70S6K from isolated rat heart tissue in response to the treatment with artemisinin (Art) (4.3µM) and rapamycin (rapa)(0.1µM) in combination (artemisinin and rapamycin) (Art+rapa) and alone in the ischaemic reperfusion model.

Figure 39b: Western blot demonstrating Phospho-p70s6k $_{(Thr389)}$ expression with artemisinin (Art) 4.3µM and rapamycin (Rapa) (mTOR inhibitor) (0.1µM) which abolishes expression induced by Artemisinin. Rapamycin (0.1µM) was also administered alone, at reperfusion and subjected to same experimental protocol. (***P<0.001 vs IR and *P<0.05 vs Art)(n=4).

Figure 40. The assessment of phosphorylated BAD expression as analysed using flow cytometric analysis upon treatment with artemisinin (Art) (4.3µM) at reoxygenation following the hypoxia/reoxygenation protocol in the presence and absence of wortmannin (Wort)(PI3K inhibitor)(0.1µM0.vValues are expressed as MEAN±SEM (**P<0.01 vs Norm,##P<0.01 vs H/R,*P<0.01 vs Art)(n=6-8).

Figure 41a: Hypothetical schematic representation of the artemisinin mediated cardioprotection in isolated cardiomyocytes and isolated perfused heart subjected to H/R injury and I/R respectively via the activation of the pro-survival signalling pathway PI3K-Akt kinase cascade and their targets BAD and p70S6K. PI3K-Akt is an important part of the RISK pathways identified by Hausenloy and Yellon (2003). Administering artemisinin (4.3µM) at reperfusion has is suggested to initiate cardioprotection by protecting against reperfusion-induced cell death. The scheme portrays the important anti-apoptotic mechanisms that have been implicated in mediating cellular survival associated with the recruitment of these kinase cascades via the PI3K-Akt cascade shown to reverse the activation of caspases and proapototic protein BAD via the p70S6K upon administering artemisinin in different settings of I/R and H/R.

Figure 42. An Image of isolated ventricular myocytes.

Figure 43. The effects of artemisinin (4.3µM) in the presence and absence of non-selective Nitric oxide inhibitor, L-NAME (100µM) and aminoguanidine (100µM) on LVDP expressed as a percentage of mean stabilisation. Hearts underwent 20 minutes of stabilisation, 35 minutes ischaemia and 120 minutes of reperfusion in the presence/absence of artemisinin (4.3µM) ± inhibitors which were administered throughout reperfusion. Results presented are Mean±SEM (n=3-8).

Figure 44. The effects of artemisinin (4.3µM), L-NAME (100µM) and aminoguanidine (100µM) on H/R as a percentage of mean stabilisation. Hearts underwent 20 minutes of stabilisation, 35 minutes of Ischaemia and 120 minutes of reperfusion in the presence/absence of artemisinin (4.3µM) ± inhibitors. Art (4.3µM) was administered throughout the 120 minutes reperfusion with/without inhibitors (n=3-8).

Figure 45. The effects of artemisinin (4.3µM) in the presence and absence of inhibitor, L-NAME (100µM) and aminoguanidine (100µM) on CF (CF) expressed as a percentage of mean stabilisation. Hearts underwent 20 minutes of stabilisation, 35 minutes ischaemia and 120 minutes of reperfusion in the presence/absence of artemisinin (4.3µM) ± inhibitors which were administered throughout reperfusion. Results presented are Mean±SEM (n=3-8).

Figure 46. Assessment of nitric oxide survival pathway in artemisinin (Art) mediated cardioprotection in isolated perfused heart model subjected to I/R. Art (4.3µM) was administered at reperfusion in the presence and absence of either L-NAME (non selective nitric oxide inhibitor)(100µM) or aminoguanidine (Amg) (iNOS inhibitor)(100µM). Results are shown as Mean±SEM. ***P<0.001 vs. IR, *P<0.05 vs. Art, #P<0.05 vs. L-NAME, ##P<0.01 vs. Art. (n=3-8).

Figure 47. MTT reductase activity used in assessing cellular viability in isolated ventricular cardiomyocytes subjected to 2 hours of hypoxia and 2 hours of reoxygenation. Where Art (4.3µM) was administered in the presence and absence of L-NAME (100µM) and aminoguaninde (100µM). ### P<0.001 vs Norm, **P<0.01 vs H/R, *P<0.05 vs Art, #P<0.05 vs H/R (n=3-8).

Figure 48. Effect of administration of artemisinin (Art) (4.3µM) in the presence and absence of L-NAME (100µM) and aminoguanidine (Amg) (100µM) on cleaved caspase-3 levels as analysed by Flow Cytometry. ***P<0.001 vs Norm, *P<0.05 vs H/R, ##P<0.01 vs. Art, #P<0.05 vs. Art. (n=4-6).

Figure 49. Effect of administering artemisinin (Art) (4.3µM) throughout reoxygenation in the presence and absence of L-NAME (100µM) on eNOS expressions as analysed using Flow Cytometry. ***P<0.001 vs Normoxia (Norm), **P<0.01 vs H/R, ##P<0.01 vs. Art. (n=4-6).

Figure 50: Effect of artemisinin (Art) (4.3µM) on iNOS expression in the presence and absence of L-NAME (100µM) and aminoguanidine (Amg) (100µM) analysed using FACS analysis. *P<0.05 vs. Normoxia (Norm), **P<vs. H/R, ##P< vs. Art (n=4-6)

Figure 51a and 51b. Nitrite and Nitrate Standard Curves. Nitrite (left) and nitrate (right) standard curves were performed according to the OxiSelect™ *in vitro* nitric oxide colorimetric assay protocol showing a directly proportional increase in the absorbance and concentration of nitrite and nitrate in control samples respectively. Results expressed as Mean±SEM

Figure 52. The effects of artemisinin, doxorubicin and the co-administration of artemisinin and doxorubicin on LVDP. Hearts underwent 20 minutes of stabilisation, 35 minutes of ischaemia and 120 minutes of reperfusion, during which the drugs were administered at the onset of reperfusion. Data is expressed as Mean±SEM. **P<0.01 vs. I/R control (n=4-8), *P<0.05 vs. I/R

Figure 53. The effects of doxorubicin (1µM) in the presence/absence of artemisinin (4.3µM) administered at reperfusion in an isolated perfused heart model subjected to I/R. Results are shown as Mean±SEM. ***P<0.001 vs. IR, ###P<0.001 vs. Art, #P<0.05 vs. Art, $$$P<0.001 vs. Dox. (n=3-8)

Figure 54. MTT reductase activity used in assessing cellular viability in isolated ventricular cardiomyocytes subjected to two hours of hypoxia and two hours of reoxygenation. Dox (1µM) was administered in the presence/absence of Art, L-NAME (100µM) and aminoguanidine (100µM). ### P<0.001 vs. Norm, **P<0.01 vs. H/R, *P<0.05 vs. Art, ***P<0.001 vs. Dox, **P<0.05 vs. L-NAME (n=3-8) (D+A+L=Dox+Art+L-NAME and D+A+Ag= Dox+Art+aminoguanidine) (n=3-6)

Figure 55. The effects of doxorubicin and inhibition of the nitric oxide pathway on cellular viability. Doxorubicin (1µM) was administered alone in the presence and absence of L-NAME (100µM) and aminoguanidine (100µM). ### P<0.001 vs. Norm, **P<0.01 vs. Dox, *P<0.05 vs. H/R. (n=3-6).

Figure 56. Effect of administering doxorubicin (Dox) (1µM), artemisinin (Art) (4.3µM), both drugs combined and in the presence and absence of L-NAME (100µM) and aminoguanidine (Amg) (100µM) on cleaved caspase-3 activity in isolated adult rat cardiomyocytes after two hours of hypoxia followed by two hours of reoxygenation (H/R) as analysed using Flow

Cytometry. Results are shown as Mean±SEM. ***P<0.001 vs. Norm, *P<0.05 vs. HR, ***P<0.001 vs. H/R, ^^^P<0.001 vs. Dox, **P<0.01 vs. Art (n=4-6). (D+A+L=Dox+Art+L-NAME) and (D+A+Ag=Dox+Art+Aminoguanidine).

Figure 57. Effect of doxorubicin (Dox) (1µM), artemisinin (Art) (4.3µM), both drugs combined and in the presence and absence of L-NAME (100µM) and aminoguanidine (Ag) (100µM) on cleaved caspase-3 activity in isolated adult rat cardiomyocytes after two hours of hypoxia followed by two hours of reoxygenation (H/R) as analysed using Flow Cytometry. Results are shown as MEAN±SEM. ###P<0.001 vs. Norm, ***P<0.001 vs. H/R. (n=4-6).

Figure 58a. Effect of administering doxorubicin (Dox) (1µM) and artemisinin (Art) (4.3µM) throughout reoxygenation in the presence and absence of L-NAME (100µM) on eNOS (Ser 1171), expressions as analysed using Flow Cytometry. ***P<0.001 vs. Normoxia (Norm), **P<0.01 vs. HR, ###P<0.001 vs. Dox, ##P<0.01 vs. L-NAME (n=4-6) (D+A+L=Dox+Art+L-NAME)..

Figure 58b. Effect of administering doxorubicin (1µM) throughout reoxygenation in the presence and absence of L-NAME (100µM) on eNOS expressions as analysed using Flow Cytometry. ***P<0.001 vs. Normoxia (Norm) (n=4-6).

Figure 59a. Effect of doxorubicin (1µM) and artemisinin (Art) (4.3µM) on iNOS expression in the presence and absence of L-NAME (100µM) and aminoguanidine (Ag) (100µM) analysed using FACS analysis. *P<0.05 vs. Normoxia (Norm), **P<0.01 vs. H/R, ##P<0.01 vs. Dox. Data expressed as mean± SEM (n=4-6).

Figure 59b. Effect of doxorubicin (1µM) on iNOS expression in the presence and absence of L-NAME (100µM) and Aminoguanidine (Ag) (100µM) analysed using FACS analysis. *P<0.05 vs. Normoxia (Norm) (n=4-6).

Figure 60. The effects of artemisinin (0.4mM), doxorubicin (1µM), L-NAME (100µM) and various combinations of the three drugs on the cytotoxicity of HL-60 cancer cells. ***P<0.001 vs. control, #P<0.05 vs. Art/Dox, ###<0.001 vs L-NAME Data presented as mean ±SEM (n=4).

Figure 61: Hypothetical schematic representation of the adjunctive therapy using artemisinin and doxorubicin in isolated cardiomyotes and isolated perfused heart subjected to H/R injury

and I/R respectively via the downstream targets of PI3K-Akt kinase cascade, Nitric oxide. Administering Artemisinin (4.3µM) and doxorubicin (1µM) at reperfusion initiated cardioprotection by protecting against reperfusion-induced cell death in cardiomyocytes. The scheme portrays the important anti-apoptotic mechanisms that have been implicated in mediating cellular survival associated with the recruitment of nitric oxide shown to reverse the activation of caspases and increase nitrite and nitrate production. Doxorubicin (1µM) and artemisinin (0.4mM) nearly doubled doxorubicin cytotoxicity in HL-60 cell line.

LIST OF TABLES

ABBREVIATIONS

Art	Artemisinin
WORT	Wortmannin
L-NAME	N_w-Nitro-L-arginine methyl ester hydrochloride
AMG or Ag	Aminoguanidine
RAPA	Rapamycin
DMSO	Dimethyl sulfoxide
TBST	Tris-buffered Saline Tween 20
PI3-K	Phosphatidylinositol 3-OH-kinase
mTOR	mammalian target of rapamycin
NO	Nitric Oxide
NOS	Nitric oxide synthase
eNOS	endothelial nitric oxide synthase
iNOS	inducible nitric oxide synthase
nNOS	neuronal nitric oxide synthase
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
IC_{50}	half maximal inhibitory concentration
EC_{20}, EC_{50}, EC_{80}	20% effective concentration, 50% effective concentration (or half maximal effective concentration), 80% effective concentration

HR	Heart rate
LVDP	Left ventricular developed pressure
CF	Coronary flow
TTC	2,3,5-triphenyltetrazolium chloride
KH buffer	Krebs Heinsleit (KH) solution
I/R	Ischaemia-reperfusion#
H/R	Hypoxia/Reoxygenation
MTT	Thiazolyl blue tetrazolium bromide
miRNA	MicroRNA
FACS	Fluorescence Activated Cell Sorter
PCR	Polymerase chain reaction
(qRT-PCR) or RT-PCR	Quantitative real-time reverse transcription polymerase chain reaction
RB	Restoration Buffer
HL-60	Human leukaemia cancer cell line HL-60
p-Akt	phospho-Akt
p-p70S6 Kinase	phospho-p70S6K

Chapter 1

1 INTRODUCTION

1.1 CARDIOVASCULAR DISEASE AND CORONARY HEART DISEASE

A Cardiovascular Disease (CVD) is one of a variety of diseases of the heart and blood circulation. CVDs, including Coronary Heart Disease (CHD) and Cerebro-vascular Disease, are currently the leading cause of death globally, accounting for about 21.9% of total deaths at present and projected to increase by 26.3% by 2030 (Ali *et al*., 2010).

The global burden of death as a result of CVD deaths worldwide is estimated to be more than all communicable diseases, maternal deaths, neonatal deaths and deaths due to nutritional disorders (Nichols *et al.*, 2014). It is also estimated to be responsible for double the number of deaths caused by cancer and approximately half of all deaths in Europe. Medical advances such as surgeries and the use of therapeutics have led to significant decrease in mortality rates as a result of CVD (Anderson *et al*., 2007; Nichols *et al*., 2013). Research has also attributed the modest changes in mortality to cardiovascular health behaviour with emphasis on diet and weight, coupled with a reduction in the prevalence of smoking to be responsible for this improvements (Roger *et al*., 2012). Despite the noticeable decline in mortality, CVD remains accountable for over 4 million deaths in Europe alone yearly (Nichols *et al*., 2014).

The prevalence of CHD and Cerebrovascular Accidents (also known as ‘strokes’ which result from a blood clot cutting off the blood supply to parts of the brain (Roger *et al.*, 2012), has resulted in this condition being a serious health concern particularly in the western world, often leading to a large proportion of deaths and physical disabilities (Nichols *et al.*, 2013).

CHD in particular has the highest mortality rate of all diseases in the UK (Schünke *et al.*, 2006) and the most common cause of premature death in the UK (BHF, 2012). Presently, one in six men and one in ten women die of CHD each year, with death rates more prevalent with age (NHS, 2014). CHD will be the leading cause of morbidity and mortality in the developing world in general by 2020 (Lopez and Murray 1997; Goldberg *et al*., 2004; Kloner and Rezkalla

2004). Although the mortality rate for CHD appears to be declining, morbidity rate appear to be on the rise with prevalence higher in lower socio-economic groups (BHF, 2012).

One of the main subtypes of CHD is myocardial infarction (MI) (also known as a 'heart attack'). MI is caused by the occlusion of one of the coronary arteries by atherosclerotic plaque formations (Reynold 2012). Coronary arteries bring blood and oxygen to the heart and a blockage in the arteries starves the heart of blood and oxygen, leading to cellular death (Cohn *et al*., 2000). CHD, angina (a sharp pain in the heart which may be explained as a result of lactic acid build), MIs and strokes have all been associated with atherosclerosis (Reynold, 2012). Atherosclerosis is a condition whereby the arteries become narrowed due to a gradual build-up of fatty deposits (atheroma) along the arterial walls, causing it to narrow and thereby restricting blood flow (BHF, 2012) (as shown in Figure 1).

Figure 1. *The build-up of fatty deposits in atherosclerosis (CHF, 2013).*

Atheroma formed in the arterial wall may rupture and break away to form blood which may block the coronary artery and deprive the heart of oxygen rich blood. Studies have highlighted the importance of inflammatory cascades as mediators in the pathogenesis of atherothrombosis (Libby, 2002).

1.2 ATHEROSCLEROSIS

Atherosclerosis is a degenerative CVD in which the arteries become narrowed or blocked, often with fat, cholesterol, calcium, fibrin and cellular waste products (Anderson *et al*., 2007). This tends to restrict blood flow, causing insufficient delivery of oxygen and nutrients to meet the

hearts demand, a condition referred to as ischaemia – the atheromas in atherosclerosis cause functional constrictions or obstructions to blood vessel, a very common pathological condition in CVD (Anderson *et al.,* 2007).

Progressive atherosclerosis manifests itself as coronary artery disease, which could lead to angina, myocardial infarction or sudden death (Reynold, 2012; Anderson *et al*., 2007; Maganti *et al.,* 2010). Currently, the most effective treatments for the management of these conditions are surgical interventions and the use of pharmacological agents, however there is no known cure (Anderson *et al*., 2007).

The most common etiological factors responsible for myocardial infarction is the presence of an atherosclerosis plaque blocking the coronary arteries as seen in the Figure 2 (Asano *et al*., 2003). This leads to the disruption of blood flow to the cardiac myocytes, causing the activation of an ischaemic cascade (Libby and Theroux 2005). During an ischaemic heart attack, lactic acid accumulates as a result of the switch from aerobic to anaerobic respiration during energy production by the myocytes, leading to an ischaemic region (Solani and Harris 2005). Consequently, Adenosine Triphosphate (ATP) levels become depleted, owing to the failure of Na^{+}/K^{2+} ATP-dependent pumps to function, and an imbalance in intracellular ion concentration within the cell occurs causing a reversal in function of the sodium-calcium exchanger (Inserte *et al*., 2004). The resultant metabolic mismatch of ions and failure of the pumps causes a calcium overload, oxidative stress, ATP depletion leading to cell death (Javadov *et al.,* 2009).

Figure 2. *Cross-sectional view of a normal coronary artery, an artery thickened due to atherosclerosis and an artery with a blood clot induced as a result of atherosclerosis (NIH, 2014)*

During the early stages of atherogenesis, leukocytes are recruited to the site of inflammation causing them to adhere to the endothelial wall (Libby 2002). Recruitment of the leukocytes is by the P and E-selectins that are upregulated in the process of atherogenesis resulting in the chronic inflammatory disorder (van Wanrooij *et al.*, 2008). This interaction between the selectins slows down the blood leukocytes and causes the integrin leukocytes to bind to endothelial cell adhesion molecules leading to the recruitment of monocytes and lymphocytes (van Wanrooij *et al.*, 2008). Monocytes within the sub-endothelial space activate matrix metaloproteinases (MMP) which degrade connective tissue within the matrix, thus inducing the release of macrophages which in turn release pro-inflammatory cytokines that cause monocytic proliferation and cytokine mediated progression of atherosclerosis (Libby 2002).

Several risk factors have been identified in atherosclerosis, with some being potentially reversible, such as hypercholesterolemia, hypertension, cigarette smoking, obesity and physical inactivity while other risk factors include age, ethnicity, family history, diabetes and gender (Libby, 2000, Schonbeck *et al.*, 2000, Collins *et al*., 2000).

1.3 MYOCARDIAL INFARCTION

As previously mentioned, myocardial infaction has been reported to occur with angina in acute coronary syndrome. Coronary artery disease and atherosclerosis are reported to have strong associations as both arteries share the same systemic environment (Bando *et al*., 2015).

Myocardial infarction (MI) (also known as heart attack) refers to the irreversible necrosis of the heart due to prolonged ischaemia (Zafari *et al*., 2015). This condition is often mistaken with cardiac arrest however in MI the prolonged limitation of blood flow to the heart is accompanied by chest pain and discomfort with the symptoms starting slowly as opposed to the sudden cardiac arrest, MI results in damage to the heart muscles and eventually heart failure/cardiac arrest if symptoms are not relieved (AHA, 2014). Heart attack is however a common cause of cardiac arrest and often requires cardiac pulmonary resuscitation (CPR) which is an emergency response to keep blood flowing to the heart until help comes (Anderson *et al.*, 2007; AHA 2014).

Major advances have recently been made in understanding the pathogenesis of acute coronary syndromes such as angina and MI (Grundy 1999). The most effective treatments for the management of these conditions presently are surgical interventions and the application of drug

therapy such as aspirin (an anti-platelet), warfarin (an anticoagulant), statins (which lower cholesterol levels), beta blockers and angiotensin converting enzyme (ACE) inhibitors are all widely accepted means of managing CHD and its various manifestations (Anderson *et al.*, 2007). Ultimately, patients with MI will require reperfusion treatments i.e. medical treatment that restores blood flow to the blocked arteries following occlusion (Verma *et al.*, 2002; Kloner and Rezkalla 2004).

1.4 ISCHAEMIA

As previously mentioned, heart disease arises through injury to cardiac tissue by an imbalance of blood supply, causing an insufficiency in oxygen and nutrients required to satisfy myocardial demand and this phenomenon is referred to as ischaemia (Rosenfeldt *et al.*, 2006).

If ischaemia persists within the myocardium for a significant duration of time, it results in a spectrum of clinical syndromes which are characterised with metabolic and ultrastructural changes that led to irreversible injury (Eltszchig and collard 2004). These distinct alterations to cells such as cellular swelling, disruption of oxidative phoshorylation, decline of intracellular pH, decrease in levels of ATP, lactic acid build up, altered metabolism and free radical mediated injury, which may all occur if the onslaught is prolonged (Allen *et al.*, 2008; Lichtig and Brooks 1974; Halestrap *et al*., 2004; Buja 2005).

1.5 REPERFUSION

Reperfusion as described in many studies, is a medical intervention administered to reduce ischaemic injury to tissues (Kloner and Rezkalla 2004; Yellon and Haunsenloy 2013). This treatment has resulted in years of success and is still an effective way of managing ischaemia and, in the long run, curbs mortality (Kloner and Rezkalla 2004). Although early reperfusion remains the best strategy to reduce ischaemic injury, restoration of blood flow to the ischaemic organ often causes a subsequent burst of reactive oxygen species (ROS), inflammatory mediators and the exacerbation of the ischaemic injury known to cause injury in the affected organ (Yellon and Haunsenloy 2013; Silachev *et al.*, 2014; Shim 2010). Reperfusion is a well documented critical procedure employed to salvage reversibly damaged myocytes, by reintroducing coronary flow (CF) to the blocked artery and reperfusing the ischaemic myocardium (Verma *et al*., 2002). Early and successful myocardial reperfusion using either thrombolytic therapy or primary percutaneous coronary intervention are proven to be the most effectively used strategies of managing myocardial infarction and improving the clinical

outcome (Yellon and Haunsenloy 2007). However, this timely reperfusion to an ischaemic area as indicated can produce two variable effects on the myocardium, i.e. in an attempt to salvage a great amount of the myocardium and thus decreasing cardiac related morbidity and mortality, it also results in a paradoxical cardiomyocyte dysfunction, a phenomenon commonly termed "reperfusion injury" (Buja 2005; Verma *et al*., 2002; Yellon and Haunsenloy 2007).

Despite its benefits, negative attributes resulting from reperfusion injury have been shown to include arrhythmias, enzymatic release, myocardial stunning followed by reversible and/or irreversible cell damage (Kalogeris *et al.,* 2012). Free radicals are also generated in ischaemia as well as reperfusion (Zweir and Talukder 2006, Kalogeris *et al.,* 2012). Previous studies have established that progression of myocardial injury is enhanced by neutrophils in our immune system and mast cells which accumulate at the site of injury during ischaemia injury and reperfusion injury (Jordan *et al.,* 1999: Jeroudi *et al*., 1994). ROS and Reactive Nitrogen Species (RNS) are believed to encourage ischaemic injury as well as reperfusion injury (Tompkins *et al*., 2006). An experimental model of 3 weeks chronic exposure to hypoxia in Sprague Dawley rats was shown to promote the generation of ROS *in vivo* which was accompanied by hypoxia induced cardiopulmonary changes. Lung xanthine oxidase was shown to be elevated from day one of exposure to hypoxia which was followed by vascular thickening and ventricular hypertrophy that contributed to the development of cardiac pulmonary hypertension (Hoshikawa *et al*., 2001). In ischaemic conditions ATP is broken down to purine hypoxanthine via oxidation or proteolytic cleavage, thus reducing xanthine oxidoreductase to xanthine oxidase which in turn results in the formation of ROS and hydrogen peroxide (Pritsos, 2000). Xanthine oxidoreductase is an enzyme usually present in the myocardium as a rate-limiting step in purine degradation to uric acid (Jankov *et al.,* 2008).

The generation of free radicals in the mitochondria have been associated with free radical scavengers such as superoxide dismutase and glutathionine peroxidase (Poyton *et al*., 2009, Venditti *et al*., 2013). Superoxide dismutase is an enzyme existing as a free radical scavenger in a normal functioning myocardium. However when the enzyme cleaves as a result of stress induced from both ischaemia and reperfusion, the production of ROS is increased (Venditt *et al*., 2013).

Other factors that have been linked to myocyte necrosis include ROS, dysregulation of mitochondrial Ca^{2+} homeostasis, mitochondrial fragmentation and cytochrome c release in the myocytes (Hom *et al.,* 2009, Webster, 2012,). These factors play a key role in pathologies and

are extremely damaging to the myocardium, despite their independent roles in disease, mitochondrial matrix Ca^{2+} overload can lead to the enhanced generation of ROS, triggering the opening of the permeability transition pore, and release of cytochrome c which leads to apoptosis (Brookes *et al*., 2004).

The sustained injury is often reversible since the myocardium can normally tolerate brief periods (of up to 15 minutes) of severe or total ischaemia without resultant cardiomyocyte death; this is often encountered in the clinical situations of angina, vasospasm and angioplasty and is not associated with concomitant myocyte cell death (Yellon and Baxter 2000). Increasing the duration and severity of ischaemia leads to greater cardiomyocyte damage (Verma *et al*., 2012).

Despite the return of blood flow to an ischaemic region, cardiac stunning often follows. Cardiac Stunning is a process whereby the contractile function of a section of the myocardium is affected, resulting in a visible reduction in heart contraction (Pomblum *et al*., 2010). Evidence has shown that free radical damage occurs upon reintroducing flow and thus myocardial stunning is viewed as a sub lethal form of oxyradical-mediated reperfusion injury (Bolli 1990). Studies have also shown that the generation of these oxyradicals which causes the sarcoplasmic reticulum dysfunction, also leads to calcium overload, which in turn exacerbates the damage initiated by the oxygen species (Pomblum *et al.*, 2010). Although myocardial stunning is spontaneously reversible, studies continue to investigate and focus on the benefits and the mechanism of stunning, though reperfusion therapies have proven by far more relevant in terms of attenuating myocardial ischaemia/reperfusion injury (Pomblum *et al*., 2010).

Reperfusion, which is also a potent stimulus for neutrophil activation and accumulation, results in marked endothelial cell dysfunction, changes in intracellular calcium homeostasis (which play an important role in the development of reperfusion injury), altered myocardial metabolism (which in turn may contribute to delayed functional recovery) and production of more oxygen free radical (Verma *et al.*, 2002). These effects are characterised as reperfusion injury which is also associated with microvascular dysfunction such as impaired endothelium, leukocyte plugging in capillaries and plasma protein extravasation in post capillary venules (Carden and Granger 2002).

1.6 OXYGEN-DERIVED FREE RADICALS

ROS are a group of chemically reactive ions, radicals and molecules derived from oxygen (Hancock *et al.,* 2001). Free radicals are constituently present within cells and can be produced by a number of mechanisms the majority is formed within the mitochondria during reoxygenation forming such molecules as superoxide anion an oxygen derived free radical (Bollisetty and Jaimes 2013; Stowe and Camara 2009) .

Male Sprague Dawley rat hearts that are chronically exposed to hypoxia show increased levels of xanthine oxidase hypoxanthine, a pathway known to generate oxidative stress *in vivo.* Hoshikawa *et al.* (2001) measured lung phosphatidylcholine hydroperoxide in the hypoxia-exposed rats as a marker of oxidative stress of the lung tissue and showed hypoxia led to an increase in ROS (Hoshikawa *et al.,* 2001).

During normal physiological conditions within the myocardium, xanthine oxidoreductase is present principally in the dehydrogenase form and acts as rate-limiting step in purine degradation to uric acid (Jankov *et al.,* 2008). However within ischaemic conditions ATP is broken down to the purine hypoxanthine, a substrate for xanthine oxidoreductase, and reversible oxidation or irreversible proteolytic cleavage causes the conversion of xanthine oxidoreductase to xanthine oxidase which is responsible for the detrimental formation of ROS and hydrogen peroxide (Pritsos 2000).

Prior to ischaemia the myocardium will contain endogenous free radical scavengers such as superoxide dismutase which exhibit strong antioxidant capacity preventing ROS mediated injury (Rahman 2007, Rodrigo *et al*., 2013). Changes during ischaemia and reperfusion cause these enzymes to be unable to withstand the higher ROS stress and therefore are able to produce adequate protection from the increased ROS production (Rahman 2007). Oxygen-derived free radicals are formed to destructive levels during the reoxygenation of the ischaemic region of the myocardium (Zweier *et al.,* 1994). Prior studies have demonstrated reoxygenation leads to a significant increase in oxygen derived free radicals that have many effects, one being the inhibition of contractile function in the heart (Zweier and Talukder 2006). However ROS does possess functional attributes within the body and participates in a range of different roles such as hormone biosynthesis, cell signalling and microbial killing (Rada and Leto 2008). The balance between the production and scavenging of ROS leads to homeostasis. However the accumulation of ROS is well documented in cellular damage (Stowe and Camara 2009).

A study using wild type mice and tumor necrosis factor receptor-associated protein 1 (TRAP1) knock out mice, (TRAP1 is an essential mitochondrial chaperone which is induced in rat hearts following I/R) showed upon 12 hours ischaemia and 1 hour reperfusion (I/R injury). TRAP1 overexpressed hearts were shown to generate decreased levels of ROS and ameliorate myocardial dysfunction causing delays in mPTP opening (Zhang *et al*., 2015).

Llacuna (2009) also showed the the generation of ROS increased over time, with significantly levels of ROS detected upon 30 minutes ischaemia, levels of oxidative stress peaked after 1 hour in this study male C57BL/6 mice subjected to partial hepatic ischaemia. With prolonged levels of ischaemia, endogenous protective proteins/genes may be compromised thus the use of antioxidant based strategies to preserved the level of managanese superoxide dismutase (MnSOD) protective genes which has been shown to improve the integrity of the liver (Llacuna *et al*., 2009).

Research has also shown that exogenous ROS scavengers and antioxidants can decrease the amount of ROS available in cells, thus attenuating the detrimental effects of ROS *in vitro*. Conversely, lowering the levels of oxidative stress may sometimes be beneficial in organisms where ROS is responsible for cellular signalling. The balance of ROS and antioxidants in this situation is optimal (Poljsak *et al.,* 2013).

1.7 METABOLISM CHANGES

Research has been able to establish the precise sequence of biochemical events leading to myocyte cell death including the metabolic derangement (Ferrari *et al.,* 1998). During ischaemia, the increased rate of glycolysis causes the accumulation of lactic acid thus causing the intracellular pH to drop significantly (Kalogeris *et al*., 2012). With a rapid decrease in the amount of adenosine triphosphate (ATP) available, the Na^+/K^+ ATPase is inhibited leading to a rapid rise in intracellular [Na^+] leading to changes in Ca^{2+} in the cell (Halestrap *et al*., 2007).

1.8 CALCIUM OVERLOAD

The high levels of intracellular Ca^{2+} is caused as a result of the activity of Na^+/Ca^{2+} antiporter being inhibited (which usually pumps Ca^{2+} out of the cell) (Halestrap *et al*., 2007). Calcium overload is stimulated following ischaemia immediately after reoxygenation and has been shown to lead to the development of cellular contracture and death (Vaselle 2004).

Changes in the level of Ca^{2+} regulation has been shown to be critically important in both the mechanical dysfunction and arrhythmogenesis associated with congestive heart failure. Cytosolic calcium overload can result from Ca^{2+} influx across the sarcolemma via the cation channels opening, the actiavation of the Na^+/H^+ exchanger causing an influx of Na^+ , or through the inactivation of the Na^+/K^+ -ATPase or the Na^+/Ca^{2+} exchanger. This leads to the release of calcium from the sarcoplasmic recticulum or other endogenous stores. The Ca^{2+} overload is often accompanied by hyperactivation and, consecutively, hypercontracture in cells. In tissue however, the process does not end there hypercontracture will lead to cell rupture thus increasing Ca^{2+} overload as shown in Figure 2b (Piper 2000).

This item has been removed due to 3rd party copyright. The unabridged version of the thesis can be viewed in the Lanchester Library Coventry University.

Figure 2b. Schematic diagram of the mechanistic overview of the calcium paradox (Piper 2000).

Abnormally high levels of calcium have been shown to have a number of different negative effects such as the activation of calcium dependant proteases and phospholipases that are able to cause significant damage to the sarcolemma and may even lead to cellular death (Shigekawa and Iwamoto, Halestrap *et al.*, 2007).

1.9 CELL DEATH

Cell death is an essential process within the body to allow for the removal of cells whose activities are no longer required this can be due to age or through injury (Fulda *et al*., 2010). Cell death can be divided into categories namely, necrosis, apoptosis and autophagy (Fulda *et*

al., 2010). Studies have recently investigated the relationship between myocardial ischaemic injury and the major modes of cell death, such as oncosis and apoptosis.

1.10 NECROSIS

This form of ATP independent cell death is characterised by the swelling of the organelles and entire cell after which the cell membrane becomes more permeable with the release of cellular contents causing extensive tissue injury which is associated with an intense inflammatory response (Proskuryakov *et al*., 2003). Necrosis is stimulated by stress such as ischaemia which evokes a large necrotic death of the cardiomyocytes (Fulda *et al*., 2010). What was thought to be one of the defining features of necrosis is the cells which undergo this process do by a chance occurrence however; recent studies have shown that necrosis is an active form of cell death with clear signs of regulation (Berghe *et al.,* 2014, Okada and Mak 2004).

Studies have defined the existence of two different oxidation-mediated necrotic pathways, an ATP-dependent apoptosis blockade, and the ATP independent process which involves the interactions of multiple regulatory factors (Sancho *et al*., 2006). A study using cultured mouse proximal tubular cells showed that the extent of cell death was proportional to the amount of ATP content and independent of mechanism of cell death, with an increase in cell death expected with decreased availability of ATP (Lieberthal *et al.,* 2013). However apoptosis is the active process (ATP dependent) while necrosis is an organised primary cell death process independent of ATP (Apraiz *et al.,* 2012).

1.11 APOPTOSIS

Is defined as a caspase dependent programme cell death (Fulda *et al*., 2010). It occurs under physiological conditions however it is most desirable under pathological circumstances (Apraiz *et al*., 2011). Typical morphological characteristics that accompany apoptosis or programmed cell death include plasma membrane blebbing, cell shrinkage, chromatin condensation and fragmentation (Vermeulen *et al.,* 2005). It is also accompanied by changes in caspases which trigger apoptotic cell death in cells and tissues (Vanden Berghe *et al.,* 2010)

As previously mentioned, during apoptosis, caspases are cleaved and PARP (a nuclear enzyme that is activated by DNA strand breaking) inactivated, which preserves cellular ATP despite significant DNA damage (Abraham 2011, Roos and Kaina 2006). Altered intracellular calcium

levels may also regulate oncotic cell death leading to cells swelling to an abnormally high size due to the failure of the permeable membranes ionic pumps (Fink and Cookson 2005).

Plasma membrane (PM) blebs are dynamic cell membrane protrusions which are normally initiated by a combination of events usually extracellular triggers that involve local disruption of membrane actin cortex interactions, leading to rapid protrusion of the PM as a result of the cell internal hydrostatic pressure (Fackler and Grosse 2008; Charras 2008). During the final stages of apoptosis, marked changes in cell morphology occur such as contraction and membrane blebbing, this has now been recently linked to caspase mediated activation of ROCK 1 (rho-associated coiled coil containing protein) (an effector protein responsible for the regulation of the actomyosin cytoskeleton which promotes contractile force generation and plays a role in cell motility, metastasis and angiogenesis) (Coleman 2001). Blebs have thus been implicated in cellular injury, apoptosis, cytokinesis, and cell movement in healthy cultured cells at particular stages of cell movement (Barros *et al.,* 2003; Rath and Olson 2012).

It has been suggested in the past that ischaemia alone can trigger apoptosis after prolonged occlusion without reperfusion (Borutaite and Brown 2003). However, more recently, it is proposed that myocardial apoptosis and necrosis are primarily triggered during reperfusion (Zhoa and Johansen 2002). It is a highly regulated process therefore a target for pharmacological inteventions (Fulda *et al.,* 2010). Apoptosis is characterised by a series of regulated biochemical process that leads to programmed cell death as seen in Figure 3.

Figure 3. *A general overview of the apoptotic pathways (Dabbagh and Rajaei 2013).*

This process of apoptosis is initiated by the presence of stimuli such as ROS and hydrogen peroxide which distinguishes it from necrosis which is thought to be traditionally stimulated via acute cellular injury (Elmore 2007). Apoptosis can be divided into two main pathways intrinsic and extrinsic (Park *et al.*, 2015). The extrinsic pathway involves the activation of a death receptor located in the extracellular surface of the cell by apoptotic stimuli (Elmore 2007) (as shown in Figure 3). The extrinsic pathway is a major pathways for caspase activation with apoptosis initiated via death signal ligation in the cell membrane, a death inducing signalling complex (DISC) is formed after Fas-associated death domain (FADD) and procaspase-8 as illustrated in Figure 4 (Keoni and Brown 2015).

The intrinsic death pathway is however managed by the mitochondrial action in response to G protein-coupled receptors stimulated by environmental stresses like deoxyribonucleic acid (DNA) damage leading to the release of cytochrome c, nutrient deprivation, hypoxia, and intra-mitochondrial proteins such as apoptosis inducing factor, second mitochondrial derived activator of caspades and endo nuclease G (Borutaite *et al.*, 2003; Fan *et al.*, 2013). The collective factors described earlier proceed to influence the activity effectors caspades like caspase 3 and execute the apoptotic cell death (Elmore 2007). Cytochrome c, has also been shown to binds with Apaf-1 and ATP in the cytosol form a complex known as the apoptosome this triggers the initiation of pro-caspase 9 (Keoni and Brown 2015)(shown in Figure 4). In the intrinsic apoptotic pathway, caspase activation is shown to be closely associated with the permeabilisation of the outer mitochondrial membrane by proapoptotic members of the B-cell lymphoma protein-2 (Bcl) family, mitochondrial lipids, proteins and components of the permeability transition pore usually in response to proapoptotic signals or cytotoxic stimuli that cause the disruption of the outer mitochondrial membrane. With the disrupted membrane, mitochondrial apoptogenic proteins such as cytochrome c, Second mitochondria-derived activator of caspase/direct inhibitor of apoptosis-binding protein (Smac/DIABLO), Omi/HtrA2 (mitochondrial serine protease that is released into the cytosol during apoptosis to antagonize inhibitors of apoptosis), apoposis inducing factor (AIF) and endonuclease G found between the inner and outer mitochondrial membranes are released. Upon reaching the cytosol, these apoptogenic proteins trigger the cell death by promoting caspase activation or by acting as caspase-independent death effectors (Fulda and Debatin 2006).

Caspases are synthesised as inactive proenzymes activation by apoptosis causes the cleavage of essential cellular substrates, including poly (ADP-ribose) polymerase and lamins, thus

participating heavily to the distinctive morphological changes seen within apoptosis. The caspase family main activating factor is cytochrome c release by the mitochondria through apoptotic stimuli (Borutaite *et al.,* 2003). Caspases involved in apoptosis in humans that have been classified as initiators or effectors, the initiators include caspase 2, caspase 8, caspase 9 and caspase 10) while the effectors are caspase 3, caspase 6 and caspase 7) (Wesche *et al.,* 2005). The release of cytochrome c triggers caspase-3 activation via the apoptogenic proteins forming an apotosome complex (Fulda and Debatin 2006). Caspase-3 is then activated by caspase-9 thereby causing cellular and biochemical events of apoptosis to occur (Fulda and Debatin 2006).

Several caspase inhibitors however have been identified to decrease apoptosis, in sepsis lethality apoptosis for example it is used as a potential therapeutic target (Wesche *et al.,* 2005). Caspase inhibitors are also commercially available, examples include Boc-D-fmk and Z-VAD-fmk, which function by inhibiting the activation of certain initiator or effector caspases however they are required in very high toxic concentration to be effective (50µM) (Caserta *et al.,* 2003, van Noorden 2001). Other non-toxic options do exist for example Q-VD-OPh (quinolyl-valyl-O-methylaspartyl-[-2,6-difluorophenoxy]-methyl ketone) which is a true pancaspase inhibitor and is effective at significantly lower concentrations (50µM) which is non-toxic *in vivo* but capable of crossing the blood–brain barrier (van Noorden 2001, Chauvier *et al*., 2007). These inhibitors cause a decrease in matrix metalloproteinase (MMP) and generation of ROS, this mitochondrial amplification loop of caspase activity is suggested to be an important response to cardiotoxic treatment (Fulda and Debatin 2006).

A study has tested the effectiveness of Q-VD-OPh during reperfusion by inducing stroke in both male and female poly ADP ribise polymerase (PARP) (protein involved in DNA repair) knockout mice, results however showed Q-VD-OPh only significantly reduced infarct size in PKO and wild type, stroke-induced females, with no effect observed in males (Liu *et al.,* 2008).

Mocanu et al. (2000) also investigated the effectiveness of non-selective caspase inhibitor (Z-VAD·fmk), caspase-8 inhibitor (Z-IETD·fmk), caspase-9 inhibitor (Z-LEHD·fmk) and caspase-3 inhibitor (Ac-DEVD·cmk) following I/R injury. Results showed a decrease in infarct size with drug treatment during early reperfusion which was accompanied by a decrease in the levels of caspases during reperfusion thus suggesting caspase inhibition during early reperfusion protects myocardium against lethal reperfusion injury as shown in Figure 4. (15)

Figure 4. *A cell-centric view of apoptosis and apoptotic cell death inducing strategies (Apraiz et al., 2011)*

Bcl-2 (B cell ymphoma 2), Bcl-x_L,(B cel ymphoma extra large),Bax (apoptosis regulator Bax aso known as B-cl2 ike protein 4), VDAC (voltage-dependent anion channel), GD3 (acidic glycosphingolipid ganglioside),TBID (truncated BH3-interacting domain death agonist), Smac/Diablo (Second mitochondria-derived activator of caspase/direct inhibitor ofapoptosis-binding protein), APAF (Apoptotic protease activating factor 1), ICAD/CAD(Inhibitor of Inhibitor of Caspase-activated DNase/Caspase-activated DNase compex)

The extrinsic and intrinsic apoptotic processes are both accompanied by similar biochemical, morphological and common phosphotidylserine externalisation processes (Apraiz *et al.*, 2011). These processes occur due to the increase in intracellular Ca^{2+}, although not unique to apoptosis alone (may also occur during cytochrome c release, caspase activation or DNA fragmentation) has been shown to inhibit translocase activity and activate a scramblase (a non- specific, bidirectional lipid molecule) which leads to spontaneous distribution of phospholipid in the plasma membrane (Apraiz *et al.*, 2011).

The apoptotic signal in the extrinsic pathway is triggered by a ligand binding to a complimentary cell surface receptor the tumour necrosis factor receptor (TNFR) which activates the TNF cell death pathway. Depending on the scenario, other death receptors such as CD95/Fas/Apo1 activated by the Fas ligand or the death receptor 4 and 5 (DR4/5 activated by the Apo2 ligand (Apo2L) otherwise known as TNF-Related Apoptosis-Inducing Ligand (TRAIL) may also bind to their respective ligands and induce cell death (Apraiz *et al.*, 2011).

These adaptor proteins however require the death effector domain in order to recruit and activate the cell death effectors, initiators such as caspase 8 and 10. Ligand mediated death receptor activation does not always induce apoptosis however caspases are considered prime mediators of apoptosis (Apraiz *et al.,* 2011).

Intrinsic pathway for example are associated with mitochondrial damage, recent research has also implicated the involvement of other organelles such as the endoplasmic recticulum, nucleus and lysosomes (Danial and Kosmeyer 2004, Ferri and Kroemer 2001). This process is primarily driven as a result of damage from Ca^{2+} release leading to impaired oxidative phosphorylation and opening of the mitochondrial permeability transition pore. The membrane permeability leads to the release of pro-apoptotic proteins such as apoptotic inducing factor (AIF), endonuclease G, Smac/DIABLO, Bax and Bak and HtrA2/Omi (Elmore 2007, de Bruine and Medema 2008). Bak and Bax are both members of the pro-apoptotic Bcl-2 family of protein however Bcl-2 proteins do no operate at a mitochondrial level only (Danial and Kosmeyer 2004, Brunelle and Letai 2009). Intrinsic apoptotic pathways may also be triggered by the nucleus triggered intrinsic pathways primarily involving DNA damage where protein-modifying enzyme poly (ADP-ribose) protein (PARP)-1 plays a vital role (Ha and Snyder 2000). It is proposed that PARP-1 mediated cell death is as a result of energy depletion as well as AIF translocation from the mitochondria (Ha and Snyder 2000).

1.12 CYTOCHROME C

Cytochrome c is principally recognised within the mitochondria as a key constituent of ATP synthesis however this molecule can be released into the cytosol and trigger programmed cell death (Apraiz *et al*., 2011). Cytochrome c release and cytochrome-c mediated apoptosis are managed by an intricate network of different molecular systems the most predominant the Bcl-2 family (Ow *et al.,* 2008). Cytochrome c release in the mitochondrial intermembrane space is a significant contributing factor of apoptosis within the majority of cells throughout the body. It can therefore be judged that the release of this molecule is as important to the process of apoptosis as the activation of caspases.

When cytochrome c is present within the cytosol, it binds to Apaf-1, and induces the oligomerisation of Apaf-1·cytochrome c complex in a dATP/ATP-dependent manner (Jiang and Wang 2000; Liu *et al.,* 1996; Zou *et al*., 1997). This allows for the recruitment of the initiator caspase, caspase-9, to the complex which induces procaspase-9 auto-activation (Mei

et al., 2010; Zou *et al.,* 1999). Activated caspase-9 has been shown to specifically cleave downstream caspases such as caspase-3 and caspase-7 that constitute major caspase activity in apoptotic cells (Mei *et al*., 2010; Thornberry & Lazebnik 1998; Brentnall *et al*., 2013).

In recent years, the role of the mitochondria in apoptotic cell death has been much debated. It is believed that the mitochondria of apoptotic cells undergo permeability transition (Fulda *et al.,* 2010; Fan *et al*., 2013; Marchetti *et al.,* 1996). The mitochondrial permeability transition pore (mPTP) as it is formally defined is a nonspecific channel located in the inner mitochondrial membrane (imm) (Halestrap and Brenner 2003). The molecular composition of this structure is still debated but it is believed by some to have three core components a voltage-dependent anion channel adenine nucleotide translocator, cyclophilin D exhibits peptidyl-prolyl cis-trans isomerase activity (Javadov *et al.,* 2009). After an ischaemic episode, the process of reperfusion has been experimentally shown to lead to the opening of mPTP via oxidative stress which is followed by the loss of ionic homeostasis and ultimately death by necrosis (Halestrap *et al.,* 2004).

The metabolism and ionic homeostasis of the heart are greatly perturbed leading to the opening of the mPTP. Reperfusion alone can not induce significant mPTP opening however causes myocardial stunning does. This is suggested to occur as a result of the increased injury caused by the free radical damage (Halestrap and Pasdois, 2009). mPTP located in the inner mitochondrial matrix opens as a result of calcium triggered conformational changes (Javadov *et al*., 2009; Halestrap and Richardson 2015). A high concentration of Ca^{2+} in the matrix, followed by high oxidative stress and high adenine nucleotide depletion (characteristics assoiated with the irreversible ischaemia-reperfusion injury) have been shown to collectively lead to the opening of the mPTP as shown in Figure 5 (Halestrap and Richardson 2015).

Proteins such as the Bcl-2 family and hexokinases associated with the outer mitochondrial membrane (omm) have been shown to regulate the mPTP opening via their interactions with inner mitochondrial proteins such as the imm Adenine Nucleotide Translocase (ANT), phosphate carrier (PiC) and FoF1 ATP synthase at contact sites (Murphy *et al*., 2005; Crow *et al*., 2004). Membrane potential may independently affect the opening of Ca^{2+} opening thus suggesting that mPTP is a voltage gated pore (Halestrap and Richardson 2015). Cells exposed to excessive amounts of Ca^{2+} undergo mPTP and then necrosis (He and Lemasters 2002). Pathological conditions such as I/R and heart failure have been associated with altered levels

of cardiomyocyte Ca^{2+} homeostasis , this is suggested to occur as a result of the inadequate oxygen available to the cardiac tissue thus causing a decrease in ATP levels too (Javadov *et al*., 2009). This altered Ca^{2+} homeostasis (Ca^{2+} overload), oxidative stress from reperfusion injury combined with other factors such as high phosphate and low adenine nucleotide concentrations induce the formation of nonspecific mPTP imm (Javadov *et al.*, 2009; Halestrap and Richardson, 2015). mPTP is clearly critical in regulating cell death, recent genetic studies have shown VDAC is an essential component of mPTP and attributed a regulatory (rather than structural) role to ANT while phosphate carriers were shown to play critical roles in mPTP formation thus questioning the molecular identity of the pore (Javadov *et al.*, 2009). Cardiac pathology condition such as I/R have shown the inhibition of mPTP opening by analogs of cyclosporine A or sanglifehrin A which are responsible for the mediation of ROS accumulation via mitochondria targeted antioxidants (Javadov *et al*., 2009). Opening of mPTP causes uncoupling of the mitochondria and swelling of the matrix which leads to rupturing of the omm and ultimately cell death (Javadov *et al*., 2009) as shown in Figure 5.

Figure 5. *Showing the involvement and role of CyP-D, ANT, PiC and FoF1 ATP synthase and that of the outer mitochondrial proteins in regulating the opening of mPTP activity at contact sites. Figure also shows the involvement of the outer and inner mitochondrial protein in regulating the pore as well as the presence of outer membrane protein, VDAC at this contact site which may be more important for their role in the creatine phosphate shuttle (Halestrap and Richardson 2015).*

1.13 STRATEGIES IN MI

Reperfusion is undoubtedly key in the management of MI as it leads to a reduction in morbidity and mortality rates, however, this process is time dependent. By decreasing the time of onset of symptoms to reperfusion and choosing optimal reperfusion strategies, it will undoubtedly enhance survival for patients (Zhang and Huo 2011).

1.14 CARDIOPROTECTION

Endogenous protective mechanisms have been demonstrated in several animal models and in isolated human cardiomyocytes. In ischaemic preconditioning, repeated short cycles of ischaemia (mechanical occlusion) and reperfusion are administered prior to the onset of ischaemia and have been shown to limit myocardial injury and provide the myocardium with resistance to subsequent ischaemic insult (Skyschally *et al.,* 2008; Tomai *et al*., 1999). It is considered post conditioning when the brief periods of ischaemia and reperfusion occur after the onset of reperfusion. Post conditioning has been shown to protect the myocardium and significantly reduce infarct size in several studies (Skyschally *et al.,* 2008; Zhoa 2009).

As previously mentioned, the myocardium is the source of innate endogenous protective mechanisms that are shown to be stimulated during reperfusion and to exert beneficial effects. However, they are very often found to be insufficient in preventing the deleterious effects of reperfusion injury itself. Viable endogenous protective mechanisms include adenosine production, opening of ATP-sensitive potassium channels (K_{ATP}), as well as release of nitric oxide (Verma *et al*., 2002).

Research has shown that the protective effects of preconditioning are transient and last for greater than 2 hours however despite the promise of preconditioning and post conditioning, the experimental findings cannot be directly extrapolated to humans due to the fact that no clinical study can meet the conditions of experimental studies on preconditioning in which infarct size is the end-point (Tomai *et al.,* 1999).

1.15 PRECONDITIONING

Ischaemic preconditioning was first described in 1986 by Murrey *et al*. and involves episodes of sub lethal ischaemia which is able to delay necrosis upon myocytes during a subsequent lethal ischaemic insult. The studies were done on a canine model with 4 consecutive periods of 5 minutes coronary occlusion which were shown to decrease infarct size by nearly 75%

when accompanied by a 40 minutes occlusion (Tomai *et al*., 1999). On a cellular level the underlying mechanism is still elusive however preconditioning does result in the activation of several different receptor families such as delta-opioid, alpha-adrenergic (Banerjee *et al*., 1996), bradykinin and adenosine (Mullane & Bullough 1995). Stress signals seem to play a prominent role in ischaemic preconditioning through the activation of signalling molecules such as mitogen-activated protein kinases, protein kinase C elevated synthesis of numerous protective proteins and the opening of ATP-sensitive K^+ channels (Tomai *et al.,* 1999; Healy *et al.,* 2015).

Ischaemic preconditioning induces protection against I/R injury however, ischaemic preconditioning requires direct interference with the target tissues blood supply, limiting its clinical utility and feasibility because the process can be mechanical and/or pharmacological (Healy *et al.,* 2015). Preconditioning in one tissue, such as the kidneys, has been shown experimentally to confer protection on distant organs such as the heart (Przyklenk *et al.,* 1993). Pre-conditioning has however been shown to be feasible only during electric cardiac procedures thus encouraging the search for pharmacological cardioprotective agents that will target the reperfusion phase (du Toit *et al.,* 2009).

1.16 POST CONDITIONING

Brief episodes of ischaemia within the early stages of reperfusion termed as post conditioning. This process has been suggested to be just as effective as preconditioning and has been shown to protect against I/R injury. Post conditioning perfomrend immediately or within 30 minutes after reperfusion is referred to as rapid post conditioning whereas it is considered delayed pos conditioning if post conditioning is performed hours or days later (Zhoa 2009). One study assessed the beneficial effects and showed post conditioning limits infarct size and improves endothelial function in isolated rat hearts subjected to I/R injury (Galagudza *et al.,* 2004). Another study showed post conditioning showed a decrease in infarct size by 44% comparable with the protective effect of preconditioning (Zhoa *et al.,* 2003).

The protective mechanism of post conditioning in the myocardium has been confirmed by several studies (Zhoa and Vinten-Johansen 2006), in pigs (Iliodromitis *et al.,* 2000) as well as in human trials (Straat *et al.,* 2003). The protection offered as a result of pre-conditioning/post-conditioning is potent, but limited to a narrow therapeutic time window (Simon 2014). In

animal experiments, a complex signal transduction cascade was identified which results specifically in a reduction of reperfusion injury, this prosurvival kinases are called the RISK (reperfusion injury salvage kinase) pathway (to be discussed in later sections). There is evidence that also shows that both ischaemic pre- and post-conditioning protects the myocardium against subsequent myocardial injuries in patients with coronary artery disease (Skyschally *et al.,* 2008).

1.17 PHARMACOLOGICAL AGENTS IN MI

Mechanism of pharmacological pre-conditioning, which implores the use of pharmacological agents to counteract the burst in ROS and Ca^{2+} overload are also effective strategies in limiting ischaemic injury (Alvarez *et al.,* 2014). Pharmacological agents such as adenosine, a non selective adenosine receptor agonist resulted in the same infarct size reduction as was observed in preconditioned hearts in rabbit hearts this study by Liu et al. (1999) was the first to show adenosine's therapeutic potential (Sivaraman and Yellon, 2013). Following the success of animal studies, the acute myocardial infarction study of adenosine (AMISTAD) proceeded to investigate the effect of adenosine in a randomised population of 236 patients treated with thrombolysis, results showed 33% relative reduction in infarct size in the group that received adenosine and 67% reduction in infarct size amongst patients with anterior myocardial infarction (MI) however for this study infarct sizes was determined by single-photon emission computed tomography (SPECT) and the end point was a reduction in clinical events (Sivaraman and Yellon, 2013). Efficacy of this study was limited to patients with anterior MI, however an increase in adverse clinical events was present in patients with non anterior myocardial infarction, this is a limitation of the first study as it only evaluated clinical events as the end point. The AMISTAD II study studied 2,118 patients with evolving ST-segment elevation in myocardial infarction patients undergoing reperfusion therapy and found no significant difference in the primary end point between placebo group (17.9%) and the adenosine treated groups (16.3%) (Ross *et al.,* 2005). The negative results observed were suggested to be from administering AMP579 for an average time of 0.37 hours prior to reperfusion whereas in animal studies, evidence for infarct size reduction with AMP579 is robust in terms of timing, dosage, and duration of administration. Another explanation for the negative results is that an infusion was given with no bolus dose which suggests that the concentration of AMP579 was likely not sufficient at the point of reperfusion and for several hours after. A_{2b} activity has been shown to play a crucial role in cardioprotection by AMP579 however, A_{2b} is shown to be a low-affinity receptor that requires a high dose of agonist to result

in activity this suggests a negative result may be as a result of the blood concentration of AMP579 varying greatly in patients as Ross *et al.* (2005) stated, steady state concentration of the drug was achieved only in 4 to 6 hours. With several adenosine receptor subtypes being investigated, such as A_1, A_{2a}, A_{2b}, and A_3 subtypes, there is a great potential in their possibility of reducing the side effects of adenosine and produce better outcomes (Sivaraman and Yellon, 2013).

As mentioned earlier, therapy for MI is myocardial reperfusion by mechanical or pharmacological preconditioning. Animal studies have shown pharmacological agents such as bradykinin and adenosine administered prior to reperfusion limit infarct size whereas clinical trials have shown administering glucose–insulin-K^+-therapy (GIK) 10-11hrs after the onset of symptoms drastically improved the clinical outcome of patients by 62% (Quintana *et al.*, 2003). However due to the unequivocal positive results GIK is not currently used in clinical practice (Doenst *et al.*, 2011).

The Pol-GIK-trial (Polish- GIK trial) showed in a low-risk MI patient population, study had to be terminated prematurely due to the increase in mortality in GIK-treated patients (Dirksen *et al.*, 2007). There were substantial differences between different studies particularly with regard to the time at which symptoms appear and start of treatment, and regarding the composition of the GIK cocktail and the duration of treatment. At present clinical studies support the effectiveness of insulin-glusoce infusion in diabetic and acute MI patients admitted to a surgical intensive care unit for at least 3 months but treatment needs to be accompanied by meticulous glucose regulation. Whether this effect can be effectively translated into routine practice is questionable (van der Horst *et al.*, 2002)

Positive results have been obtained with clinical trials using agents such as CSA (cyclosporine A). CSA administered at the onset of reperfusion was established as a pharmacological inhibitor which has the ability to prevent mPTP opening and reduce myocardial infarct (MI) size in animal models of acute ischaemia reperfusion injury (Ong *et al.*, 2015). This has improved the translatability of mPTP inhibition and as a novel therapeutic approach in the clinical setting however, CSA is not a specific mPTP inhibitor (Ong *et al.*, 2015).

Other drugs such as levosimenden with its K_{ATP} channel-opening properties administered as a pre-treatment has been shown to effectively reduce infarct size where it was proposed that pre-treatment with levosimenden could be useful before elective cardiac surgery while the post

treatment may be useful in acute coronary artery events (du Toit *et al.,* 2009). Clinical trials recommended administering levosimendan a day prior to surgery (preoperative therapy initiation), this significantly improved the heart parametres in patients undergoing cardiac surgery. Study concluded the unique inotropic and cardioprotective properties of levosimendan if administered prior to surgery can provide sustained effects for several days which can help reduce complications in the postoperative period (Toller *et al.,* 2015).

Research has shown that adenosine, opiates and bradykinins are endogenously released in cells and acts on cell membrane receptors. Results from these studies suggested that selective pharmacological antagonists of the δ-or κ-opioid receptor are important triggers/mediators of protective response in both acute and delayed preconditioning. All these agents have been shown to block IPC and activate the G-protein coupled pathways resulting in a potent protective effect (Gross 2003).

In addition to opioids having potent cardioprotective effects in various animal models, clinical studies using isolated human atrial trabeculae that have undergone hypoxic preconditioning showed by administering the δ-opioid agonist, DADLE protects the atrial tissue from I/R injury (Tomai *et al.,* 1999). Opioid receptors are thought to play important roles in protecting the human myocardium during times of stress (Gross 2003). Results from both animal and human studies suggest the stimulation of δ- or κ-opioid receptors produce both acute and delayed cardioprotective effect (Gross 2003). Same way pharmacological agents have been associated with cardioprotection, other agents have been associated with drug induced cardiotoxicity.

1.18 DRUG INDUCED CARDIOTOXICITY

As it is becoming apparent that different drugs have the ability to cause serious adverse effect or even death (Force and Kerkela 2008). Drug induced cardiotoxicity has become a critical factor that needs meticulous attention when administering drug in treating different pathologies. Cancer patients seem to survive a lot longer due to the efficacy of drug treatment especially the anthrayclines however treatment is usually hampered by drug induced cardiomyopathies especially with popular anthracyclines such as result of drug induced cardiotoxicity as popular presented upon treatment with popular doxorubicin (Gharanei *et al.,* 2014). Cardiotoxic risks are therefore a major concern especially with the potential of artemisinin as a possible chemotherapeutic agent (Crespo-Ortiz and Wei 2011, Nam *et al.,* 2007, Lai *et al.* 2013)

In an attempt to establish links in the differential expression of apoptosis-related miRNAs in the cardiomyocytes subjected to I/R injury we investigated the expression profiles of certain popular miRNA's such as miRNA1, miRNA27a, miRNA133a, miRNA133b and miRNA155 against artemisinin treated cardiomyocytes.

1.19 MICRO RNAS (miRNAS)

Currently, there is a limited understanding regarding miRNA-mediated apoptotic pathways in myocardial injury. Although several studies have showed miRNAs to be important regulators of apoptosis and cardiac injury. Depending on the nature of the targeted genes, miRNAs can either be pro-apoptotic or anti-apoptotic (Yang *et al.,* 2009).

Research has shown a large class of small noncoding RNAs which are ~22 nucleotides in length in animals, known as miRNAs, function as important regulators of several biological processes by modulating gene expression (Salic and Windt, 2012; van Rooj 2011). These miRNAs are produced by two RNase III proteins- Drosha and Dicer (Ha and Kim 2014). Their main function is post-transcriptional gene regulation upon perfect complementarity based on G•U pairing between the target 3′-untranslated region (UTR) and the first 8 nucleotides of miRNA (He and Hannon 2004). They also play a major role in many other biological processes such as embryogenesis and differentiation, organ development and function, cancer, immunity and infection and toxicology/xenobiotics response (He and Hannon 2004). The functional characterisation of miRNAs is dependent on the identification of miRNA target genes. The dysregulation of miRNAs is related to a variety of disease particularly cancer where they are known to play dual functions as both tumor suppressor and oncogenes (Ha 2011; Ha and Kim 2014). They are believed to be readily circulating in blood, hence the proposition of miRNAs as novel biomarkers of disease state, especially for the detection and identification of cardiac injury allowing them to emerge as regulators of ischaemia reperfusion (Weiss *et al*., 2012).

Recently miRNAs have been implicated in the pathogenesis of cardiovascular diseases (such as heart failure, apoptosis and hypertrophy) and their altered expression profiles are currently being developed as novel diagnostic and therapeutic markers against cardiovascular disease and cancer (Ha *et al.,* 2011; Salic and Windt, 2012; Papageorgiou *et al*., 2012, Port and Sucharov, 2010).

A study showed, the differential expression of miRNA-1 is closely related with I/R injury in a rat model (Tang *et al*., 2009) and is well documented to be a biomarker for predicting acute

myocardial infarction in humans (Salic and Windt, 2012; Tang *et al*., 2009). miRNA-27a plays a pivotal role in the pathogenesis of cardiac hypertrophy and dysfunction and often implicated in cancer (Wang *et al.*, 2011; Mertens-Talcott *et al.*, 2007). Both human and rat models subjected to I/R injury, left ventricular dilation or myocardial fibrosis presented downregulation in miRNA-1 and miRNA-133 thus promoting myocyte hypertrophy which could lead to heart failure (Rooij *et al.*, 2008).

1.20 MEDICINAL HERBS AND CARDIOPROTECTION

Extract from medicinal herbs are frequently used in attenuating ischaemia reperfusion injury. Extracts such as *Psidium guajava* L. and *Limonium wrightii* have expressed cardioprotective effects in isolated perfused rat hearts where they significantly attenuated ischaemic injury and improved myocardial dysfunction after reperfusion (Yamashiro *et al.,* 2003). These drugs are believed to function via their radical-scavenging actions (Yamashiro *et al.,* 2003).

Other traditional herbal drugs have been evaluated in terms of heart failure and myocardial energy metabolism and these studies have suggested that cardiac function in patients with heart failure can be improved by inhibiting energy production from fatty acid metabolism and/or glucose metabolism (Apostolova and Victor 2015, Vogel *et al.*, 2005. This was found to be a common feature of the herbal medicines studied in this study, as they mostly contain a multi-sugar which can be metabolised into single sugar units of sugar that can be beneficial to the myocardium and potentially produce the cardioprotective effects (Ma *et al.,* 2011).

Other antioxidants have also been screened from medicinal plants such as ethanolic extracts from Curcuma longa L-EtOH Phyllanthus emblica L-EtOH and Piper rostratum Roxb-EtOH for protection against doxorubicin induced cardiotoxicity in patients receiving doxorubicin treatment which showed a dose dependent cardioprotectiveness (Wattanapitayakul *et al.*, 2005). Various other herbal extracts have proved highly effective in curing/managing a variety of diseases. Drugs such as aspirin and digitalis have also become a mainstay in the treatment of acute/recurrent pericarditis and several cardiovascular disorders respectively (Imazio and Adler 2013; Juneja *et al*., 2012).

Chinese medicinal plants such as *Carthamus tinctorius L.* (safflower), are widely used in clinical practice in managing angina pectoris. The anti-ischaemic effects of the purified version of *C. tinctorius* (ECT) has also been investigated extensively both *in vivo* and *in vitro* (Mohanty *et al.,* 2012). More recent discoveries of Chinese herbal drugs such as artemisinin and their

derivatives have also proven a mainstay therapy against a variety of diseases especially malaria in chloroquine resistant countries and has shown much potential in attenuating myocardial injury (Sun *et al*., 2007). However, artemisinin has been proven to attenuate I/R injury and post infarct myocardial remodelling in rats by down regulating NF-kB pathway after MI. Recently, artemisinin was also found to suppresses cardiac hypertrophy in primary cultured rat cardiac myocyte via the nuclear factor (NF)-κB signalling pathway, by inhibiting genes involved in the pathogenesis of myocardial hypertrophy and heart failure (Xiong *et al.,* 2010).

1.21 ARTEMISININ

Artemisia annua Linn is a Chinese medicinal plant, extracted and purified from the sweetworm wood (Shen *et al*., 1984). Artemisinin is the active ingredient which contains an endoperoxide moiety (Bilia *et al.,* 2014; Krishna *et al*., 2008; Crespo-Ortiz and Wei 2012). The endoperoxide moiety in the artemisinin's structure has enabled it to become the primary drug of choice presently against the *P.falciparum* and *P.vivax* malaria parasites (Nakase *et al*., 2008). Artemeether (a derivative of artemisinin) has been shown to be effective as Quinine in the treatment of cerebral malaria in children (van Hensbroek *et al.,* 1996).

Artesunate, artemether and arteether are the more widely used derivatives which are used for oral, intramuscular, rectal and intravenous administration (van Agtamael *et al.,* 1999). They are mostly used in malaria treatment, where they are universally converted to dihydroartemisinin (DHA) in the body, an active metabolite (Melendez *et al.,* 1991).

Artemisinin's have a wide range of biological functions as demonstrated in recent years ranging from anti-malarial, anti-tumour, anti-viral and anti-inflammatory activities (Lai *et al*., 2005).

1.22 ARTEMISININ AND DERIVATIVES

The artemisinin's (artemisinin, DHA and arteether) however have been first reported to display immunosuppresiveness *in vivo* in the 1980's, where they were reported to suppress the antigen-specific IgM- and IgG-mediated antibody responses and other humoral responses in mice (Tawfik *et al*., 1990; *Sun et al*., 1991; Shen *et al.,* 1984). Further studies have also shown their ability to exert anti-inflammatory action against other disease conditions such as bowel disease (Yang *et al*., 2012).

Due to the artemisinin's potent anti-inflammatory and immunomodulatory effects in the treatment if rheumatoid arthritis and systemus lupus erythematosus, these therapies provide

more efficacious, specific and less toxic effects in the short and long term compared to the standard therapies thus offering much promise (Kell 2009; Yang *et al.,* 2012; Choy and Panayi 2001).

Artemisinin's have also been implicated in allergic inflammation (Ho *et al*., 2013). Artesunate was proposed to attenuate experimental allergic asthma via the inhibition of the PI3K/Akt signalling cascade and NF-κB activation in 6 to 8 week old female BALB/c which were sensitized and challenged with ovalbumin to develop airway inflammation (Cheng *et al.,* 2011; Ho *et al.,* 2013).

1.23 ANTI-VIRAL ACTIVITY OF ARTEMISININ

There has been a strong demand for alternative anti-viral agents with mechanisms of action different from the current anti-cytomegalovirus (CMV) therapies. Presently used anti-CMV agents such as ganciclovir, foscarnet, and cidofovir, target the viral DNA polymerase (Efferth *et al*., 2008). Although very efficient in targeting the DNA, elongation in the viruses results in a dose dependent adverse effect leading to drug resistance, bone marrow suppression in patients, tetragenicity and so on (Mocarski *et al*., 2007; Harter & Michel, 2012). Artemisinin yet proves itself as an agent with versatile pharmacological effects with its artemisinin's bioactivity and that of its semisynthetic derivative artesunate, which has demonstrated even broader versatility. Artesunate amongst other effects has been shown to inhibit viruses, such as human cytomegalovirus (HCMV) and some of the *Herpesviridae* family (herpes simplex virus type 1 and Epstein-Barr virus), hepatitis B virus, hepatitis C virus and bovine viral diarrhoea virus as shown in Figure 7 (Efferth *et al.,* 2008).

1.24 ANTI-INFLAMMATORY AND AUTOIMMUNE EFFECTS OF ARTEMISININ

Autoimmune diseases such as multiple sclerosis and rheumatoid arthritis are both lifelong disabilities accompanied with a reduced life expectancy. The high safety and tolerability profile of artemisinin and its derivatives adds to their attractiveness as a chosen therapy in a variety of clinical setting (Efferth *et al.,* 2008). There is increasing evidence that artemisinin and its derivatives have immunosuppressive effects (Hou *et al.,* 2014).

1.25 ARTEMISININ AS AN ANTIMALARIAL AGENT

Nearly two billion people are at risk of developing malaria all over the world and an estimated one million die of the disease annually from malaria related illnesses (Wang *et al*., 2010). Despite substantial advancements in antimalarial treatment, concerns were growing with the use of popular quinines and quinoles in the treatment of malaria (Hara *et al*., 2007).

In the 1960's, Chinese scientists demonstrated that artemisinin (*Qinghaosu)* could clear the blood of malarial parasites more quickly than any other drug (Dondorp *et al*., 2011). Qinghoasu was widely used all over China during the 1980s and it took nearly a decade before it reached the global stage (Dondorp *et al*., 2011). The worldwide adoption of the artemisinin as efficient treatment to malarial parasite has contributed to significant reductions in morbidity and mortality due to malaria in many parts of the world including parts of Africa (Uhlemann and Fidock 2012).

Presently, the artemisinin's and their derivatives are widely administered as the mainstay combination therapy against malaria parasites due to their rapid eradication of the parasite, efficiency in treating otherwise resistant parasites and clearance at various stages of the parasites development as shown in Figure 6 (Krishna *et al*., 2006). This has led to continuous efforts in developing newer derivatives of artemisinin's with higher efficacy for anti-malarial treatment that will overcome potential drug resistance (Ho *et al.,* 2014).

Previous experimental models claim anti-malarial action of artemisinin involves the formation of free radicals via cleavage of the endoperoxide bond (-c-o-o-c) in artemisinin's structure to form a carbon based free radical intracellularly which results in the formation of free radical oxygen species (ROS) leading to death of the *Plasmodium* parasites (Lai *et al*., 2005; We *et al.,* 2014). Wang *et al.* (2010) suggested that the artemisinin's effect as an antimalarial drug is through its direct and specific mitochondrial activation. In order to prevent parasites from developing resistance to this drug WHO called for a halt in the administration of artemisinin as a single pill and requested pharmaceutical companies to sell the drugs as combination treatments and not monotherapies (Krishna *et al*., 2006 Nosten and White 2007).

1.26 ARTEMISININ COMBINATION TREATMENTS

Artemisinin based combination treatments (ACTs) are now generally accepted as the best treatments for uncomplicated falciparum malaria (Nosten and White 2007). However recently,

there have been reports on the emergence of partial resistance in western Cambodia to artemisinin, which requires tighter and stricter measures to be placed (Uhlemann and Fidock 2012). Adhering to the strict rules of the WHO in administering artemisinin will help prevent parasite resistance. Additional interventional control measures such as optimal deployment, coverage, and midcourse adjustment of containment strategies also remain essential (Dondorp *et al.*, 2012).

1.27 ANTIMALARIAL CARDIOTOXICITY

Another major concern, which has constantly had the global stage, is the threatening cardiotoxicity with the use of a variety of antimalarial drugs (Hara *et al.*, 2006). Effectiveness of drugs such as quinines in treating severe malaria in children has been challenged due to poor compliance and over 40% fatality (Achan *et al.*, 2011; PrayGod *et al.*, 2008). Parasites have been reported to have developed resistance to existing antimalarial drugs which leds to drawbacks in the attempt to eradicate malaria (Mwai *et al*., 2009). Quinines have presented cardiotoxic risks such as impaired left ventricular contractility, heart failure and increased risk of death (Lipshultz *et al*., 2004; PrayGod *et al*., 2008).

It is well established that different antimalarial drugs present associated neurotoxicity and cardiotoxicity in experimental models (Balint, 2001; Kinoshita *et al*., 2010). With the ACT however no study has reported significant cardiac effects at therapeutic doses (van Vugt *et al.*, 1999; Gupta *et al*., 2005). A more recent study even showed artemisinin's cardioprotective effect in the myocardium by showing its ability to alleviate myocardial injury and attenuate post infarct myocardial remodeling after MI (Sun *et al*., 2007; Gu *et al.*, 2012).

1.28 ANTI-CANCER ACTIONS OF ARTEMISININ

Besides the known action in anti-malarial treatments, artemisinin and its derivatives artesunate and dihydroartemisinin have shown potent *in vitro* and *in vivo* anti-cancer activity against a variety of cancer cell lines (Hou *et al.*, 2008, Singh *et al*., 2011). The observed pleiotropic effects in cancer cells with artemisinin treatment include apoptosis, inhibition of angiogenesis, growth inhibition via cell cycle arrest and the disruption of cell migration (Firestone and Sundar 2009).

1.29 TARGET RECEPTORS IN CANCER

Iron is essential in cell growth and proliferation in both in normal and cancer cells (Heath *et al.,* 2013). Cancer cells take up a large amount of iron in comparison to non-tumouric cells, with some cancer cells shown to express 5-15 times more transferrin receptors compared to normal cells (Nakase *et al*., 2007, Singh *et al.,* 2001 and Lai *et al*., 2005) which give rise to the selective cytotoxicity of the agents (Crespo-Ortiz *et al.,* 2011 and Lai *et al.,* 2005). Iron successfully binds to the transferrin (a glycoprotein attached to iron molecule) which in turn binds to the transferrin receptor (TfR), an important step in cellular uptake, pumping iron out of cells and re-using the TfR (Daniels *et al.,* 2012). Cancer cells divide uncontrollably due to the multiple signals received and over expressed transferrin receptors (TfR) which are usually upregulated on cellular surfaces of several cancer types (Wang *et al*., 2010).

Artemisinin has been shown to readily conjugate with TfR enabling the internalisation of artemisinin with the receptor bound transferrin leading to the formation of free radicals which can kill tumour cells (Oh *et al.,* 2009; Lai *et al.,* 2005). Artemisinin-tagged transferrin is highly selective and potent and ferrous iron is shown to enhance the anti-cancer efficacy of the artemisinin's up to about ten-fold (Efferth *et al*., 2004; Lai *et al.,* 2005).

Figure 6*. The different pharmacological actions of artemisinin (Ho et al., 2014)*

1.30 ANTICANCER AND CARDIOTOXICITY

Anthracyclines are one of the most effective anticancer treatments ever developed but their use is hampered by their cumulative dose limiting cardiotoxicity (Ng *et al*., 2006). Drug induced cardiotoxicity is very common in cancer therapy and is emerging as a critical issue amongst cancer survivors (Rachi *et al.,* 2010). Drug induced cardiomyopathies ranging from arrhythmias, myocardial infarction to heart failure (Adao *et al*., 2013).

Anthracyclines such as doxorubicin are quite efficient in killing cancer cells however treatment is often accompanied with chronic side effects which may even be developed years after terminating therapy (Kalyanaram *et al.,* 2002). It is also well documented that anthracylines may exacerbate co-morbid effects in patients suffering from heart disease and may be responsible for a considerable amount of morbidity and mortality in patients (Adao *et al*., 2013; Daniels *et al*., 2012).

1.31 ANTICANCER ACTIVITY OF DOXORUBICIN

Doxorubicin is a widely used antineoplasmic drug, effective in a wide range of cancers both haematological and solid tumours (Ichikawa *et al*., 2014; Shi *et al.,* 2011). Doxorubicin has well documented cumulative and dose dependent cardiac toxicities (Zhang *et al.,* 2009; Ichikawa *et al*., 2014). Cardiotoxicity is a major limiting factor in anticancer therapy (Yeh et al., 2004). Manifestations of doxorubicin range from decreased left ventricular developed pressure (LVDP) to irreversible cardiac dysfunction, which occurs in more than a third of patients (Ramond *et al*., 2008; Kumar *et al.,* 2012). Toxicity which may be acute or chronic cardiomyopathy, both leading to cardiac dysfunction, myopathy and eventually heart failure or death (Wallace 2003; Yeh *et al.,* 2004).

The mechanisms of doxorubicin induced cardiotoxicity have been studied extensively and the primary mechanisms responsible for the efficacy of doxorubicin in killing rapidly dividing cancer cells is shown to be related to DNA damage. Studies have suggested that the observed oxidative damage in the myocardium seen upon doxorubicin administration is due to iron-mediated ROS activity (Ichikawa *et al.,* 2014). Specifically, the reduction of doxorubicin activity by nicotinamine adenine dinucleotide (NADH) dehydrogenase in mitochondrial respiratory complex I, to form the superoxide radical (Volkova and Raymond 2011). This is the

widely accepted primary mechanism of doxorubicin toxicity, though iron chelator studies contradict this theory (Shi *et al.*, 2011). Studies have also shown mitochondrial DNA lesions, dysregulation for calcium handling, adrenergic dysfunction and selective inhibition of cardiomyocyte-specific gene expression to play a pivotal role in doxorubicin induced cardiotoxicity (Zhang *et al.*, 2009).

It is suggested that cardiomyocytes may be much more sensitive to oxidative stress caused by doxorubicin due to their high reliance on oxidative substrate metabolism (Volkova and Rusell 2011). The pathogenesis and prevalence of doxorubicin induced cardiotoxicity remains problematic thus encouraging more studies to detect, prevent and treat/manage the condition (Volkova and Rusell 2011).

Clinical trials in the past have administered doxorubicin in combination with dexrazoxane (a cardioprotective drug) and it was shown to reduce the cardiotoxic effects of doxorubicin. Dexrazoxane acts by diminishing tissue damage resulting from extravasation of doxorubicin in cells (Jordon *et al*., 2009). However, dexrazoxane has since been withdrawn from the market due to the signs of it causing acute myelogenous leukaemia in paediatric patients and secondary malignancies in older patients (Tebbi *et al.*, 2007). Prior to that the clinical implementation of dexrazoxane labelled it as the first and only proven antidote in anthracycline extravasation (Jordon *et al*., 2009).

Dexrazoxane has been shown to reduce cardiotoxicity in adults and children with a range of tumor types (Swain and Vici 2004). However, cancer occurs mostly in elderly people and its predisposition increases with advancement in age (Eschenhagen *et al.*, 2011). Pharmacological improvements in cancer therapy has led to an increase in long life expectancy for many patients, however, treatment related comorbidities have become an issue especially in long term cancer survivors (Bovelli *et al*., 2011). Clinical trials investigating cancer treatments however, presently under-represent vulnerable groups such as the older patients and patients with significant comorbidities. Given their mechanism of action, oncology patients are generally susceptible to drug induced cardiotoxicity, coupled with age, comorbidities, concomitant medication and prior exposure to chemotherapy and radiotherapy. The magnitude and risk of adverse effects increases amongst these group of patients thus raising the need to develop new cancer therapies that target treatment at a more molecular level (Eschenhagen *et al.*, 2011).

Adjuvant chemotherapy using the monoclonal antibody, trastuzumab which targets HER2 has been shown to significantly improve patient survival rates however trastuzumab, when given in combination with anthracyclines, has been associated with asymptomatic and symptomatic left ventricular dysfunction which may lead to significant cardiac morbidity leading to the premature discontinuation of trastuzumab therapy (Nohria 2013). Some of these studies remain ambiguous and inconclusive while some adjunctive therapies used result in a decrease in the cytotoxic effects of the anthracycline, making it less efficient for the purpose it was initially administered for hence the intensified pressure to develop a cardiotoxic free anthracycline (Simunek *et al*., 2008).

Cardiac toxicity is one of most feared side effects of anticancer agents, so the gain in life expectancy due to anticancer therapy might be countered by increased mortality due to cardiac problems such as heart failure, myocardial ischaemia, arrhythmias, hypertension, thromboembolism and generally poorer life expectancy as a result as other has been previously pointed out (Bovelli *et al.,* 2010). Doxorubicin, a potent and effective anti-cancer agents is associated with adverse cardiac effects, including cardiomyopathy and progressive heart failure (Doyle *et al*., 2005). It has also been shown to have other off target effects which may lead to serious consequences in non dividing cells such as the brain, with the effects reported to be less severe in bone marrow cells due to their rapidly dividing nature (Minotti *et al*., 2004). Although cardiomyocytes divide, they have a limited capacity to regenerate and off targets effects that affect the heart may lead to serious life threatening conditions (Minotti *et al.,* 2004). In a study by Gharanei et al. (2013), doxorubicin was shown to cause a time dependent reduction in the haemodynamic function of the heart as well as cause an increase in the infarct size to risk ratio in both naïve and I/R setting.

1.32 CELL SIGNALLING PATHWAY

Cellular homeostasis is key to regulating normal cellular functions, it has previously been shown to be dependent on the ability of cells to perceive and respond to stimulus appropriately. Extracellular and intracellular signals are translated in the cell and converted to cellular response such as cell division, cell cycle arrest or apoptosis. This is shown to occur via a complex network of signaling cascades which regulate cellular activities such as cellular survival metabolism, growth, proliferation, apoptosis and cell migration. These signals are transmitted to the cells first via cell surface receptors for appropriate ligand binding leading to conformational changes and subsequent phosphorylation/dephosphorylation of proteins that

result in the activation or inhibiton of downstream effectors of that pathway. This is presented as a response depending on the activated/inhibited pathway (Engelmann *et al*., 2006). It is paramount to investigate cellular signalling associated with drug effects in order to understand their applicability.

1.33 REPERFUSION INJURY SIGNALLING KINASE (RISK)

Studies by Hausenloy *et al*. (2005) have established that the recruitment of prosurvival kinases, Akt and extracellular signal-regulated protein kinases 1 and 2 (ERK1/2) commonly termed RISK pathway, confers powerful cardioprotection against myocardial I/R injury making the pro survival kinases, important targets for cardioprotection. Further studies have that the shown RISK pathway involves the activation of PI3K/Akt or extracellular regulated kinase (Hausenloy *et al*., 2007). PI3K/Akt and ERK 1/2 are parallel branches of the RISK pathway that are not independent of each other. Treatment in rat heart models subjected to I/R injury and treatment with ERK1/2 inhibitor, PD98059 has previously been shown to lead to the phosphorylation and activation of Akt (Hausenloy *et al.*, 2004).

PI3K-Akt pathway is a popular cell survival pathway that has been shown to catalyse the phosphorylation of inositol-containing lipids, known as phosphatidylinositol's which are converted to phosphatidylinositol-3, 4, 5-trisphosphate, an important second messenger molecule. Previously, implicated in cell growth, survival, motility and metabolism in normal and cancer cells (Courtney *et al.*, 2010; Oudit and Penninger, 2009; Ye *et al*., 2010). The cardioprotective role of PI3K-Akt pathway has also been shown in the myocardial I/R setting (Jonassen *et al.*, 2004; Mangi *et al*., 2003; Tsuruta *et al*., 2002) and in preconditioning setting (Hausenloy and Yellon 2003). Upon activation of the PI3K cell survival pathway, its downstream targets are phosphorylated. This includes the serine/threonine kinase Akt (also known as PKB). The translocation of Akt occurs on Thr 308 on the membrane, which is necessary for Akt activation to occur. However, studies have shown that for maximal activation of this pathway, an additional phosphorylation at Ser473 by the rapamycin-insensitive mTOR complex is required (Sarbassov *et al*., 2008).

Activation of the RISK pathway, can promote cell survival by phosphorylating pro survival molecules such as Akt, endothelial nitric oxide synthase (eNOS) and p70S6K (Hausenloy *et al*., 2004; Mocanu and Yellon 2003). These studies also showed a decrease in the activation of apoptotic proteins such as p53, caspases and members of the Bcl family, BIM, BAD and BAX.

Another important pathway, responsible for normal cell proliferation, survival and differentitation is the Mitogen-activated protein kinase (MAPK). Although it is not part of the RISK pathway, it is related to the extracellular signal-regulated kinase (ERK) pathway. ERK/MAPK pathway has led to the development of several pharmacological inhibitors used in the treatment of cancer. MAPK/ERK are shown to be activated by the Raf serine/threonine kinases. The MAPK/ERK kinase (MEK) 1/2 dual-specificity protein kinases, has been shown to activate ERK1/2 an important signalling pathway for cytoprotection (Roberts and Der 2007).

1.34 DOWNSTREAM TARGETS OF PI3K-AKT PATHWAY LEADING TO THE ACTIVATION OF THE NITRIC OXIDE PATHWAY

Signaling dynamics are often influenced by a feedback mechanism. The RISK pathway explains signalling through either the PI3K–Akt and/or the MEK1/2–Erk 1/2 cascades will lead to the inhibition of apoptosis by inactivating caspases 3 and 9, inactivating pro-apoptotic proteins BIM, BAX, BAD and p53 as well as activation of endothelial nitric oxide synthase (eNOS) which in turn produces nitric oxide (a ubiquitous molecule linked with homeostasis and pathology) (Hausenloy *et al.,* 2003). Nitric oxide is a well-known cardioprotective molecule generated via the cGMP/PKG pathway, it is also a major player in the RISK (reperfusion injury salvage kinase) and SAFE (survivor activating factor enhancement) cardioprotective pathways. Tamareilli *et al.* (2011) reported the RISK and SAFE pathway interact in rats exposed to I/R injury where SAFE pathway inhibitor (AG490) was shown to abolish cardioprotection by blocking both Akt and GSK-3β phosphorylations, whereas RISK inhibitors (wortmannin or U0126) abolished cardioprotection and blocked STAT-3 phosphorylation.

Bose *et al.* (2005) investigated Glucagon-like peptide 1 (GLP-1), (a gut hormone that stimulates insulin secretion) in isolated perfused rat hearts which were previously shown to activate anti-apoptotic signaling pathways such as PI3K pathway and MAPK in pancreatic and insulinoma cells. Using infarct size as an end point, this study showed GLP-1 added before the onset of ischaemia demonstrated a significant reduction in infarction compared with the valine pyrrolidide (an inhibitor of its breakdown) This protection was abolished using GLP-1 receptor antagonist exendin (9-39), cAMP inhibitor Rp-cAMP, PI3K inhibitor LY294002, and p42/44 MAPK inhibitor UO126 and results confirmed that GLP-1 protects against myocardial infarction in the isolated and whole rat heart models via a multitude of prosurvival kinases.

Hussein *et al.* (2013) also showed that nitric oxide plays an important role in cardioprotection against myocardial I/R injury upon administering 2-C-IBMECA (an A3 adenosine receptor agonist) which conferred protection via the PI3K-Akt cell survival pathway and the MEK1/2-ERK1/2. 2-CL-IBMECA administered throughout reoxygenation significantly reduced apoptosis, necrosis, cleaved-caspase 3 activity and increased in BAD expression. The cytoprotective effect was abolished by co-administration of IBMECA with the kinase inhibitors wortmannin and UO126 thus implicating the two pathways in cellular survival.

Endogenous nitric oxide is present in normal cellular homeostasis, however it may also be generated during ischaemia from both enzymic and non-enzymic sources (Jugdutt 2003). Ischaemia- reperfusion, triggers the release or burst of ROS which is followed by a cascade of endothelial dysfunction, decreased endothelial nitric oxide synthase (eNOS) and nitric oxide (Jugdutt 2003). The release of the free radicals, ROS converts nitric oxide into the detrimental peroxynitrite thus contributing to reperfusion injury. However nitric oxide is shown to have a ubiquitous role (Andreadou *et al.,* 2015). It is also accompanied by the release of cytokines and increase in inducible nitric oxide synthase (iNOS) and excessive nitric oxide production (Jugdutt 2003). eNOS derived nitric oxide is shown to be beneficial whereas iNOS overexpression may lead to the production of peroxynitrates resulting in cardiotoxicity (Jugdutt 2003). iNOS upregulation has been implicated in cytoprotective studies in treatments resulting from the upregulation of PI3K pathway (Smart *et al*., 2006). With the role of nitric oxide in preconditioning also well documented, administering nitric oxide prior to ischaemia decreases the possibility of I/R injury by decreasing peroxynitrite formation this suggests nitric oxide may be protective against I/R injury. Knowledge of using nitric oxide in late ischaemic preconditioning has been used towards the development of novel anti-ischaemic therapy,however role of nitric oxide in post conditioning is not precisely known (Jugdutt 2003; Andreadou *et al.,* 2015).

Studies using cultured endothelial cell from adult male Sprague Dawley rat subjected to I/R injury followed by insulin treatment showed that an increase in insulin, led to the phosphorylation of eNOS via the PI3K-Akt pathway resulting in an increase in nitric oxide production and a decrease in infarct size also implicating the nitric oxide generated via eNOS as an anti-apoptotic mediator of cellular survival in this model (Gao *et al.,* 2002). As well as nitric oxide syntheisis via the PI3K-Akt pathway, other downstream target such as p70S6K have been implicated and shown to protect the myocardium by inactivating BAD expression (Steelman *et al.,* 2011). Studies have also associated pro-survival pathways with unrestricted

cellular proliferation and decreased sensitivity to apoptotic-inducing agents (Steelman *et al.,* 2011).

Previously, PI3K-Akt pathway has been shown to promote cell survival by suppressing Bax (a pro-apoptotic member of the Bcl-2 family) translocation from cytoplasm to mitochondria. Apoptotic triggers can lead to the translocation of the Bcl-2 proteins to the outer mitochondrial membrane. The interactions between pro-survival and anti-apoptotic proteins can distort the balance between cell survival and cell death (Indran *et al*., 2011).

Mitochondria are considered decisive elements for cell death via their interactions with protein kinases and nitric oxide against apoptosis, autophagy, and necrosis (Heusch *et al*., 2008). In a study investigating the involvement of the RISK and SAFE pathway using GH-releasing hormone (GHRH), inhibitors of PI3K/Akt and STAT-3 were administered to investigate the cardioprotective effects of GHRH. This resulted in a receptor mediated activation of the RISK pathway via which the phosphorylation of BAD, eNOS, PKC and GSK occurred. These downstream targets have been shown to inhibit the opening of the mitochondrial transition pore which leads to cardioprotection (Penna *et al*, 2013). Another study revealed that Na_2S therapy activated the Erk1/2 arm of the RISK pathway which leads to cardioprotection in the setting of diabetes (Lambert *et al.,* 2014).

Nitric oxide is a downstream targets of the PI3K-Akt cell survival pathway and is produced by the endothelial nitric oxide synthase (eNOS) which is a fundamental determinant of cardiovascular homeostasis (Andreadou *et al*., 2015).Three isoforms of nitric oxide synthase (NOS) are known to synthesize nitric oxide in the myocardium. eNOS and neuronal NOS (nNOS) are both constitutively expressed isoforms, regulated by cystolic concentrations of calcium and cofactors BH4, magnesium and NADPH. The third isoform, inducible NOS (iNOS), is a calcium-independent synthase and thought to be dependent upon protein transcription (Abu-Soud *et al.,* 2000). The nitric oxide isoform eNOS is known to initiate a cascade of molecular events leading to cardioprotection (Kukreja and Lei 2007).

Nitric oxide has been shown to mediate anti hypertrophic effects (Kapakos *et al.,* 2011). A study showed treatment with nitric oxide precursor L-arginine attenuated cardiac hypertrophy in hypertensive rats and also shown to improve ventricular function (Heusch *et al.,* 2008). Cardiac hypertrophy has been associated with eNOS in angiotensin II deficient mice (Brede *at al.,* 2003) whereas iNOS-overexpressing mice have been associated with mild left ventricular

hypertrophy, contractile dysfunction, and alterations in respiratory chain complexes (Heusch *et al.,* 2008).

1.35 CELL SIGNALLING PATHWAYS ASSOCIATED WITH ARTEMISININ INDUCED CARDIAC PROTECTION IN I/R AND WITH ADJUNCTIVE THERAPY

Previous research have shown that artemisinin elicits the formation of free radicals via the cleavage of the endoperoxide bond in its structure in both anti-malarial and anti-cancers studies (Golan *et al.,* 2011).

Several pathways have been implicated in a variety of systems with both artemisinin and its derivatives. Artemisinin is believed to involve a diverse array of signalling molecules, considering it can elicit broad-spectrum inhibitory effects on several major signalling pathways such as MAPK, Wnt, β-catenin, NF-κB, PI3K pathway and so on (Mirshafiey *et al.,* 2006; Xu *et al.,* 2007; Hou *et al.,* 2009; Ho *et al*., 2012; Cheng *et al*., 2013; Li *et al.,* 2013).

For example, several studies have shown artemisinin can abrogate metastasis via the NF-κB pathway (Weifeng *et al.,* 2010; Lai *et al*., 2013; Sun, *et al.,* 2011). Ho *et al*. (2012) showed artemisinin was a viable treatment on allergic asthma and suggested its anti oxidative effects were via the upregulation of iNOS.

In a rheumatoid arthritis model also, artesunate suppressed tumour necrosis factor (TNF)-α-induced production of interleukins, interleukin 1-beta (IL-1β), interleukin-6 (IL-6) and interleuking-8 (IL-8) resulting in the inhibition of pro-inflammatory cytokines, inhibition of Akt, NF-κB and p38 MAPK (Xu *et al.,* 2007). Artesunate in addition to increasing the expression of E-cadherin caused movement of β-catenin from the nucleus to the plasma membrane causing apoptosis and reduced tumour growth via the Wnt pathway (Li *et al.,* 2008). Experimental studies have suggested that artemisinin limits injury in I/R although the associated intracellular pathways remain to be elucidated (Sun *et al.,* 2007).

Pharmacological agents previously discussed have been used to initiate cardioprotection when administered during reperfusion .These studies have also confirmed the activation of one both or these pro-survival kinase cascades that comprise the RISK pathway (as shown in Figure 7a) (Mocanu *et al.,* 2005; Baines *et al.,* 1999, Diaz 2000, Gordon *et al.,* 2003).

Figure 7a. *Activation of pro-survival PI3K–Akt and Erk 1/2 kinase cascades that make up RISK pathway (Hausenloy and Yellon 2003)*

Figure 7a show the two two branches of the anti-apoptotic pathway of cellular survival "RISK pathway" a term coined by Hausenloy and Yellon (2003).

Figure 7b. *Activation of pro-survival RAS-ERK and PI3K–mTOR which respond to extracellular and intracellular signal leading to cellular survival, proliferation, metabolism and motility (Mendoza et al., 2011).*

The Ras-extracellular signal-regulated kinase (Ras-ERK) and PI3K-Akt signaling pathways shown to either act independently or may intersect to regulate each other. The two pathways are responsible for controlling cell survival and providing compensatory mechanisms that may

positively or negatively regulate each other (Mendoza *et al*., 2011). The intensity and duration of the pathway activations are regulated by the strength of the stimulus and feedback loops which are responsibe for the cross-talks between pathways (Mendoza *et al*., 2011).

The activated ERK phosphorylates cytoplasmic signaling proteins p46 such as the P90 ribosomal S6 kinase. The nuclear targets involved with the ERK pathway includes the ternary complex factor (TCF) transcription factors, which play a major role in inducing the expression of immediate early genes which produce c-Fos and c-Myc that are responsible for inducing late-response genes that promote cell survival, cell division, and cell motility. The Ras-ERK signaling described here can trigger cell cycle arrest (Mendoza *et al*., 2011).

The PI3K-mTOR pathway on the other hand regulates the cellular process by directly recruiting the growth factor receptors or indirectly recruiting docking proteins such as insulin receptor substrate or GRB2-associated binder (Mendoza *et al*., 2011). This pathways generates phosphatidylinositol 3, 4, 5 triphosphate (PIP3), which recruits Akt that is activated by 3-phosphoinositide-dependent kinase 1 (PDK1) and a second mTOR complex mTORC1, which consists of the Ser/Thr kinase mTOR, this is a known as scaffolding protein regulatory-associated protein of mTOR (RAPTOR). This leads to the phosphorylation of eukaryotic initiation factor 4E (eIF4E)-binding protein (4E-BP) and p70S6K. Both responsible for promoting cell growth and division (Mendoza *et al*., 2011).

1.36 ISCHAEMIA REPERFUSION AND ARTEMISININ

Previously, Sun *et al.* (2007), have shown that artemisinin decreases myocardial injury in the rat heart model of ischaemia reperfusion injury. Studies have also described artemisinin as a major player in a variety of pathophysiological conditions such as antimalarial therapy (Krishna *et al.*, 2006), anti-viral activity (Harter & Michel, 2012), anti-inflammatory responses (Mirshafiey *et al.*, 2006), cytotoxicity in cancer cells (Ho *et al*., 2012) and so on. Artemisinin is therefore a drug with great potential to be used in a variety of clinical settings, without the risk of cardiotoxicity or exacerbation of cardiovascular events (Kinoshita *et al*., 2010).

Gu et al. (2012) and Sun et al. (2007) studied artemisinin in the popular off target organ (heart) and showed the cardioprotective potential in a post infarct myocardial remodeling model and in I/R setting. Hara et al. (2007) also showed the K^+ ion changes that occur in the normal myocardium may instigate cardiac arrhythmias which they also suggested may affect vascular tone in the ischaemic myocardium however treatment with artemisinin resulted in the inhibition

of the delayed rectifier K^+ current, a voltage-gated K^+ current suggesting artemisinin regulates malfunctions of the potassium channel which may alleviate cardiac arrhythmia.

1.37 AIM AND HYPOTHESIS

Given the important findings that artemisinin has cardioprotective and anti-hypertrophic properties. This study hypothesised that artemisinin's cardioprotectivenesss may be associated with the recruitment of the PI3K-Akt cell survival pathway, (an important target of cardioprotection and a branch of the reperfusion injury salvage kinase (RISK) pathway), mediated via the recruitment of pro-survival kinases such as Akt, p70S6K, nitric oxide and BAD. The current study therefore aimed to examine:

(a) the effect of artemisinin in naïve hearts and in stressed conditions;

(b) the intracellular signalling pathways associated with artemisinin-mediated cardioprotection in isolated perfused hearts and isolated ventricular cardiomyocyte models subjected to I/R and H/R injury respectively;

(c) the differential expression of miRNA upon artemisinin treatment in I/R heart;

(d) the intracellular signalling pathways associated with co-administering artemisinin and doxorubicin in isolated perfused hearts and in isolated ventricular cardiomyocytes subjected to I/R or H/R injury respectively;

(e) the effect of artemisinins treatment on HL-60 cancer cells, when co-administered with doxorubicin and associated pathways.

In summary, the study aimed to investigate cell signalling pathway associated with artemisinin's cardioprotection in I/R and H/R setting while specifically investigating pro-survival kinases PI3K-Akt, its downstream targets such as nitric oxide, p70S6K and apoptotic markers such as caspase 3. In addition to that, the study investigated the potential of artemisinin as an adjuvantive therapy in ameliorating doxorubicin induced cardiotoxicity and its cytotoxicity against HL-60 cancer cell line and associated intracellular signalling.

Chapter 2

2 METHODOLOGY

2.1 ANIMALS

Adult male Sprague Dawley rats (350-400g) were obtained from Charles River (Margate, UK) and kept at the institutional animal house to acclimatise while having free access to food and water at all times. The care and use of animals were in accordance with the Guidance on the Operation of the Animals (Scientific Procedures Act 1986). The study was carried out upon obtaining ethical approval from Coventry University Research ethics committee which was regularly assessed throughout the project

2.2 MATERIALS

(3*R*,5a*S*,6*R*,8a*S*,9*R*,12*S*,12a*R*)-Octahydro-3,6,9-trimethyl-3,12-epoxy-12*H*-pyrano[4,3-*j*]-1,2-benzodioxepin-10(3*H*)-one (Artemisinin), (1S,6bR,9aS,11R,11bR)11-(Acetyloxy)-1,6b,7,8,9a,10,11,11b-octahydro-1-(methoxymethyl)-9a,11b-dimethyl-3H-furo[4,3,2-de]indeno[4,5,-h]-2-h]-2-benzopyran-3,6,9-trione (Wortmannin) (Selective irreversible PI3K inhibitor), 27-epoxy-3H-pyrido[2,1-c] [1,4]oxaazacyclohentriacontine-1,5,11,28,29 (4H,6H,31H)- pentone (Rapamycin) were purchased from Tocris (Bristol, UK), N_w-Nitro-L-arginine methyl ester hydrochloride (L-NAME) (Non-specific inhibitor of Nitric Oxide) and Aminoguanidine hydrochloride (Selective inducible nitric oxide inhibitor) both supplied from Sigma-Aldrich (Poole, UK) and dissolved in dimethyl sulfoxide (DMSO) making sure the final concentration of DMSO was less than 0.02% as this does not affect haemodynamics or infarct size (data not included) which is then stored at -20 °C. Thiazolyl blue tetrazolium bromide (MTT) was purchased from Sigma (Poole, UK). Cleaved Caspase-3 (Asp175) Antibody (Alexa Fluor® 488 Conjugate), p-AKT_{ser473}, total AKT, p-p70S6 Kinase $_{(Thr389),}$ total p70s6k, p-$BAD_{ser136,}$ total-BAD, GAPDH, iNOS, phospho-eNOS $_{(Thr\ 495),}$ eNOS Horse radish peroxide (HRP) conjugated Rabbit monoclonal antibodies and antibiotin were purchased from New England Biolabs (Hertfordshire, UK). SuperSignal West Femto® enhanced chemoluminescent substrates were purchased from Pierce (UK). Nitric oxide Assay was purchased from Cell Biolabs,Inc (San Diego,USA), the reagents, equipments and primers used for miRNA isolation were all purchased from Applied Biosystems, UK: *mir*Vana™ miRNA isolation kit (Ambion,

Applied Biosystems, UK),Applied Biosystems MicroRNA Reverse Transcription Kit and primer assay set for U6 snRNA, rno-miR-1, hsa-miR-27a, hsa-miR-133a, hsa-miR-133b and hsa-miR-155 (Applied Biosystems, UK),TaqMan MicroRNA primer Assays (Applied Biosystems, UK) and SYBR Greeen PCR Master Mix (Applied Biosystems, UK). HL-60 cells were obtained from European Collection of Cell Cultures (ECACC).

2.3 ISOLATED PERFUSED RAT HEART MODEL

Following sacrifice by cervical dislocation, the hearts were rapidly excised and placed in ice cold Krebs Heinsleit (KH) solution (118.5 mM NaCl, 25 mM $NaHCO_3$, 4.8mM KCl, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 12 mM Glucose, 1.7mM $CaCl_2.2H_2O$) <4 ^oC and pH 7.4 as described previously (Hussain *et al*., 2014).

2.4 HEART PERFUSION PROCEDURE

The aorta was cannulated and retrograde perfusion with KH buffer. The pH of the KH buffer was maintained at 7.4 by gassing continuously with 95% O_2 and 5% CO_2 maintained at a temperature of 37 ± 0.5^oC using a water-jacketed heat exchange coil and pH 7.4.

The isolated perfused heart model also known as the Langendorff model measures cardiac function in an *ex-vivo* model at a constant diastoic pressure of 8-10mmHg. The Langendorff model has evolved since 1895 when Oscar Langendorff first pioneered the technique as a means understanding the underlying physiology of the heart in terms of its contractile function, coronary blood flow and cardiac metabolism (Bell *et al.*, 2011). In most recent studies this model has been used to probe pathophysiology of ischaemia/reperfusion and other disease states as well as study the impact of pharmacological agents and their impact on intracellular signalling and in molecular biology and genetics (Bell *et al.*, 2011). In a normal *in vivo* heart, the coronary system relies on the pressure within the heart to pump blood. Whereas the Langendorff model pumps directly into the coronary system allowing constant pressure within the heart, while maintaining a steady ejection of KH buffer via the heart chambers. This allows the system to maintain pressure and flow while maintaining contractile activity (Bell *et al*., 2011; Cheung *et al.*, 2000).

2.5 MEASUREMENT OF INDIVIDUAL PARAMETERS: LVDP, HR AND CF

The left atrium was then removed and a water-filled latex balloon was inserted into the left ventricle. The balloon was then inflated to a constant diastolic pressure of 8-10mmHg. This allows left ventricular developed pressure (LVDP) to be measured. A physiological pressure transducer was connected to a bridge amp and a power lab (AD Instruments Ltd, Chalgrove, UK) which allows the LVDP, Heart rate (HR) using ECG leads. Coronary flow (CF) was measured at regular intervals by collecting the perfusate at the distal end of the perfusion cannula. Hearts maintained a steady state of CF, HR and left ventricular developed pressure.

The experiment was conducted for 175 minutes in total. In the normoxic experiments the isolated hearts were perfused for 155 minutes with KH buffer after 20 minutes stabilisation.

***Figure 8a**. Langendorff trace showing left ventricular developed pressure and HR*

In the ischaemia/reperfusion studies, hearts were allowed to stabilise for 20 minutes followed by 35 minutes ischaemia and 120 minutes reperfusion. The anterior descending left coronary artery was ligated to induce regional ischaemia (Figure 8c). This was performed using a hooked 6-0 silk surgical suture and forceps and piercing through the heart thus ligating the flow to the left coronary artery forming a snare with the thread. The thread was passed through a pipette tip and the snare tightened to initiate ischaemia (Figure 8c). At the onset of reperfusion, the flow of KH buffer was reintroduced via the removal of the pipette tip thus releasing the snare. Reperfusion was conducted for 120 minutes (Figure 8a). Below is a schematic diagram of the langendoff set up (Figure 8b) and Figure 10 shows an outline of the experimental protocol in both the normoxic and I/R protocol.

***Figure 8b**. Schematic diagram of a Langendorff set-up showing perfusion with Krebs buffer solution which is oxygenated at the top. Cannula at the bottom of the reservoir inserted into the aorta of the excised heart and perfusion is maintained at a constant pressure and flow. Adapted from <http://www.sciseek.com/search/images&search=langendorff&type=images>*

Due to the transient ischaemia, myocardial blanching becomes visible at the site of the snare and ischaemia is accompanied by a reduced flow through the heart, a drop in LVDP and HR.

2.5.1 Drug Treatments used in the langendorff protocol

To profile the effect of artemisinin on infarct size to risk, hearts were subjected to 20 minutes stabilisation, 35 minutes ischaemia and 120 minutes reperfusion where artemisinin 0-100µM was added into the heart throughout the period of reperfusion (120 minutes) in the I/R studies. In order to establish the effective pharmacological concentrations the EC_{20}, EC_{50} and EC_{80} values were calculated as 0.042µM, 0.43µM and 4.3µM. These values were used in the presence and absence of PI3-K inhibitor, wortmannin (0.1µM), inducible Nitric Oxide Synthases (iNOS inhibitor), aminoguanidine (100µM), Non-selective Nitric Oxide Synthases (NOS) inhibitor, L-NAME (100µM) and mTOR inhibitor, rapamycin (0.1µM) (Hussain *et al*., 2011).

2.5.2 Experimental groups:

2.5.2.1 I/R treated group

EC_{80} being the most cardioprotective concentration was used for the subsequent experiments with the inhibitors of PI3k-Akt, p70S6K, non-selective NOS (L-NAME) and selective iNOS (aminoguanidine). Hearts were randomly assigned to different drug treatments and allowed to stabilise for 20 minutes while being perfused with KH buffer, then subjected to 35 minutes ischaemia followed by 120 minutes repefusion where the different drug ± inhibitor combinations are administered as shown in Figure 10. One group was treated with artemisinin (4.3μM) ± wortmannin (0.1μM) or with wortmannin (0.1μM) alone which was administered throughout the period of reperfusion. Another group was treated with artemisinin (4.3μM) ± rapamycin (0.1μM) or with rapamycin alone (0.1μM) administered throughout reperfusion. Third group was treated with artemisinin (4.3μM) ± L-NAME (100μM) or with L-NAME (100μM) alone which was administered throughout the period of reperfusion and the last group was treated with artemisinin (4.3μM) ± aminoguanidine (100μM) or with aminoguanidine (100μM).

Upon completing the I/R studies, the left coronary artery was re-ligated in preparation for staining the heart with 1ml of 0.2% Evans blue in saline, allowing differentiation between viable and tissue at risk. After staining, the hearts were weighed and stored at -20^{o}C for later analysis.

2.5.2.2 Normoxic treated group

Whereas in the normoxic study, hearts were allowed to stabilise for 20 minutes followed by 155 minutes perfusion. The following procedures were carried out: For normoxic control group, hearts were stabilised for 20 minutes followed by perfusing with KH buffer for 155 minutes. In the artemisinin treated group, hearts were stabilised for 20 minutes with KH buffer only followed by perfusing with artemisinin (4.3μM: the concentration which proved most cardioprotective from our previously described experiments).

Upon completing the treatment protocols, hearts were weighed and stored at -20 °C for 2, 3, 5-Triphenyl-2H-tetrazolium chloride (TTC) staining or the left ventricle was dissected free.

2.5.3 Triphenyltetrazolium Chloride Analysis

The hearts were then cut transversely into slices approximately 2 mm thick and incubated at 37°C in 1% triphenyltetrazolium chloride (TTC) solution in phosphate buffer for 10–12

minutes and fixed in 10% formaldehyde for at least 4 hours to enhance the staining prior to analysis.

Thereafter, the heart slices were removed from formaldehyde and placed between two Perspex sheets which were compressed with bulldog clips thus maintaining pressure. The heart slices were traced onto acetate film and were traced unto the film using different coloured markers to differentiate between the viable, at risk and infarct tissue (Figure 8d). The at risk tissue stained red and the infarct tissue appeared pale (Figure 9).

Figure 8c. *Image showing a cannulated heart, snare being tightened and ischaemia induced followed by staining with Evans blue followed by transverse slicing of the heart for further analysis. Image adapted from Bell et al., 2011*

Figure 8d*. Heart slices placed between two Perspex sheets compressed with bulldog clips (Downey, 1998)*

2.5.4 Quantifying Results: Infarct size/Risk % Assessment

The acetate film was scanned into a computer to allow calculation of Infarct to risk ratio from the differenciated tissues traced. Areas of viable, at risk and infarct tissue were measured using the Image Tool program as developed by the University of Texas Health Science Centre at San Antonio, Version 8.1 (UTHSCSA).

***Figure 9**. Hearts slices showing the differenciated areas or infarct, risk and viable tissue (Downey, 1998)*

These values were then used to calculate the % infarct to risk ratio. The percentage of infarct/risk tissue was calculated for each slice. An average reading for infarct/risk percentage for each heart was obtained by averaging the percentages of the individual slices. However, the infarct size was calculated as a percentage of the area at risk (area at risk being the area that correlates with the entire myocardial perfusion bed distal to the occluded coronary artery) (Redford *et al.,* 2012). This is reported to be the most vital, reliable and reproducible way of assessing infarct size and has thus been used by several studies in understanding mechanisms behind common diseases and also towards developing and evaluating the most appropriate treatment strategies against them (Redford *et al.,* 2012). Different hearts are individualistic therefore different in size and in terms of haemodynamics and electrophysiology too. Diverse morphological differences therefore exist between the hearts sampled, particularly when measuring infarct size, this is considered a crucial limitation of the technique hence recent studies evaluate infarct size as a percentage of area at risk. However by measuring infarct size as a percentage of the area at risk, calculating infarct size thus accounts for variability within the groups in terms of dissimilarities in the actual size of the heart and size of the area occluded (ischaemia induced) (Graham *et al.,* 2001). This is the most accepted way of determining the actual infarct size and may be used for prognosis and evaluation of drug efficacy especially in

studies aimed at reducing infarct size or in studying genetic manipulation on the ischaemic tolerance of the myocardium (Graham *et al.*, 2001; Liu *et al.*, 2002).

TTC has been shown to stain viable tissue around infarction within hours of infarction whereas the more conventiona histological stain which target firbrous scars in disease conditions may require several days from time of infarction to euthanising the animal (Redford *et al.*, 2012). Thus assessing infarction based as a percentage of area at risk using TTC staining has been shown to be an effective way of investigating diseased conditions (Redford *et al.*, 2012).

The area at risk is thus a potential source of variation in infarct analysis so this study quantified it to eliminate possible variabilities and also calculated the significant difference between the experimental groups.

The data obtained was statistically assessed using the SPSS software package. The following Figure 10 illustrates the experimental protocol used in the isolated heart model;

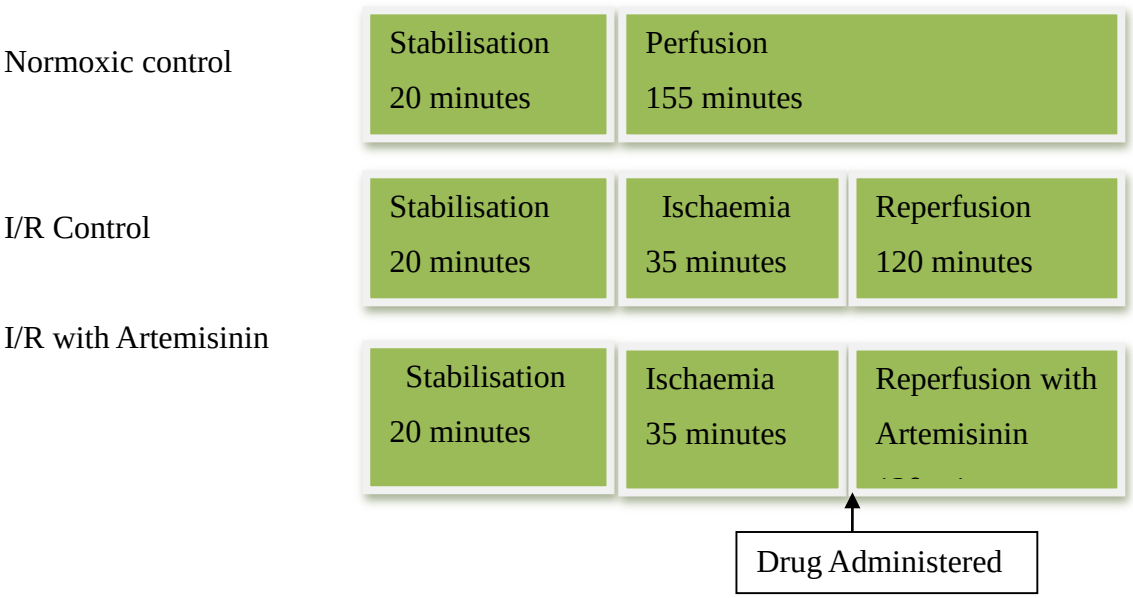


Figure 10. *An outline of the experimental protocol for infarct size assessment in the I/R and Normoxic group showing the different treatments and time*

2.6 ADULT RAT VENTRICULAR MYOCYTES ISOLATION

2.6.1 Preparations of buffers and reagents

Adult ventricular rat myocytes were isolated from Sprague Dawley rats (350-400g) by enzymatic dissociation method (Maddock *et al.*, 2002; Hussain *et al.*, 2014). After excision,

the hearts were placed in ice cold KH buffer before mounted on a modified Langendorff apparatus and perfused with modified calcium free Krebs buffer containing (in mM); 116 NaCl, 5.4 KCl, 0.4 $MgSO_4.7H_20$, 10 glucose, 20 taurine, 5 pyruvate, 0.9 $Na_2HPO_4.12H_2O$ and 25 $NaHCO_3$ dissolved in RO water. The buffer was then oxygenated with 95% O_2 and 5% CO_2 and maintained at 37^0C, pH 7.4 using NaOH.

2.6.2 Isolation of adult rat cardiomyocytes

The hearts were then perfused for 5 minutes with the calcium free buffer to clear the vessels of blood. Hearts were then perfused with a modified KH buffer and collagenase digestion buffer with low calcium concentration containing Collagenase 0.075% (Worthingtons Type II) and 4.4M $CaCl_2$, pH 7.4) at a rate of 7.5ml/min. During perfusion with collagenase the effluent was collected and re-used throughout experiment.

Following digestion, the hearts were removed from the apparatus and cut transversely into a basal and apical section. The position of the scission, at the mid-line from base to apex, was judged carefully in order to retain most of the right coronary and left anterior descending arteries in the basal section thus trimming and discarding the atria away. The ventricles were sliced and mechanically dissociated. The tissues were then incubated for 10 minutes in 25ml of digestion buffer in an orbital shaker and oxygenated with 95% O_2 and 5% CO_2. Thereafter, the suspension was passed through a nylon mesh with a pore size 400µm and centrifuged at 400 rpm for 2 minutes and the supernatant removed. The pelleted cells were re-suspended in restoration buffer (RB) (in mM 116 NaCL , 5.4 KCL , 0.4 $MgSO_4$, 10 glucose ,20 taurine, 5 Pyruvate, 0.9 $NaHPO_4$, 5 Creatine , 2% BSA, 50µM $CaCl_2$ and 1% Penstrep pH 7.4 at 37^o C) where the calcium concentration was gradually brought to 1.25 mM to avoid calcium overload. The viability of the isolated myocytes was assessed throughout by visualising the cells under a light microscope. Digested hearts with a cellular viability of below 70% were excluded. The isolated myocytes were incubated in RB (at 37 ^{o}C, 5% CO_2 for 24 hours before being used (Maddock *et al.*, 2002). Following treatment the isolated cells then underwent quantitative analysis using Fluorescence Activated Cell Sorter (FACS) or cellular viability assay using MTT reductase.

2.6.3 Principle behind FACS analysis

Protein expression within cell populations are assessed using fluorescently labelled antibodies and other fluorescent probes which can easily identify cellular patterns (Alvarez *et al.*, 2010). The flow cytometer provides high-dimensional quantitative measurement of light scatter and

fluorescence emission properties of cellular populations. In a typical analysis, cells are which are stained with fluorochrome-conjugated antibodies bind to the cell surface and intracellular targets. Within the flow cytometer, these cells are passed sequentially through laser beams which excite the fluorochromes thus measuring the emitted light, which is proportional to the antigen density (Aghaeepour *et al.*, 2013). The flow cytometry set-up consists of a laser, electronics to amplify and process signals, optical system which focuses different coloured light unto the detectors, computer and a flow cell (Alvarez *et al.*, 2010). Multiparameter flow cytometry was then developed by Paul Mullaney (also at Los Alamos) where he combined and improved the measurement of volume, light scatter and fluorescence into one.

In the current study, FACS was used for the analysis of cardiomyocytes after having undergone hypoxia/reoxygenation and different treatment protocols. In this study, intracellular staining for Caspase-3 using Alexa Fluor® 488 conjugate was used to detect myocyte cleaved caspase-3 expression, iNOS and eNOS expessions were also estimated following drug treatment. Flow cytometry is a well-known technique that can analyse and measure physical characteristics of cells as they flow in a stream of fluid through an electronic detection devise equipped with a beam of light. This allows analysis of physical and chemical characteristics of thousands of cells in a short space of time. The main components of the present day flow cytometry technique are the (1) flowing saline stream which is the fluid that transports cells through the beam of light, (2) the optic system which consists of lasers that illuminate the particles using different coloured light, filters and focuses the appropriate lights unto detectors and (3) lastly the electronic system that converts the detected light into electronic signals to be processed by computer software.

Fluorescence tagged antibodies can bind to a specific target proteins in a cell and can be used to investigate the levels of relative protein expression in a specific sample preparation. This fluorescent marker called fluorochrome fluoresces when hit by light. The molecule is excited when light of a certain wavelength loses energy and emit light of a longer wavelength excited emission. This allows the optical filters to separate lights of different wavelengths.

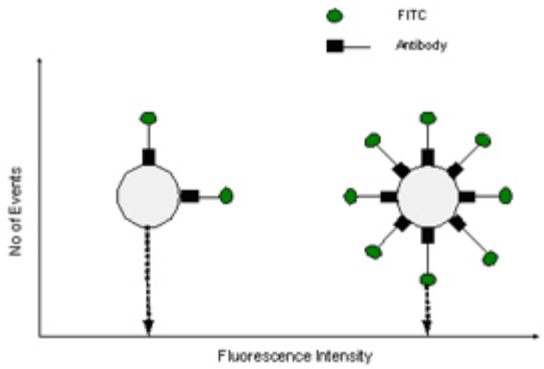


Figure 11. *Fluorescein Isothiocynate, a small sized protein that conjugates to protein through the isothiocynate group. Fluorescence emitted is proportional to binding site for the fluorescent compound.*

Light emitted can then be detected and converted into electronic signals by a computer which will give a readout of the specific fluorescence intensity detected in the sample, which corresponds to level of fluorescence tagged anti-bodies bound to the cell or the sample population investigated. The relative fluorescence value obtained corresponds to the relative protein levels in the sample of interest that were targeted by the anti-body of interest. The fluorescence intensity emitted is directly proportional to the binding sites for the fluorescent compound on the cell. So the more binding sites there are, the more fluorescence is emitted, and the higher the fluorescence intensity as illustrated in Figure 11.

2.6.4 Experimental Protocol for isolated cardiac myocytes using FACS analysis

The isolated myocytes were counted using a haemocytometer and resuspended in restoration buffer (RB) to a density of 100,000 cells/ml. 1ml of the cells was pooled to be used as normoxic control while the remaining cells were centrifuged and the pellet re-suspended in Esumi hypoxic buffer (in mM 137 NaCl, 12 KCL, 0.49 $MgCl_2$ 0.9,$CaCl_2$, 4 HEPES,20 Na lactate, 10 deoxy-D-glucose). The myocytes were then incubated in a hypoxic chamber, Galaxy 48R (New Brunswick) for 2 hours with atmosphere 5% CO_2 95% N_2 at 37^oC. Following incubation under hypoxic conditions, the myocytes were centrifuged at 500rpm for 5 minutes and the pellet was resuspended in restoration buffer. The myocytes were then assigned to the different treatment groups: artemisinin (4.3µM), artemisinin (4.3µM) ± wortmannin (0.1µM), wortmannin (0.1µM), artemisinin (4.3µM) ± rapamycin (0.1µM), rapamycin (0.1µM). The concentrations of inhibitors used are based on concentrations used in previous unpublished results of previous investigations in the lab. The myocytes then underwent reoxygentaion for 2 or 4 hours. Upon completing reoxygenation the cells were then assessed either for cellular viability using MTT (Thiazolyl blue tetrazolium bromide) or flow cytometric analysis for the assessment of p-BADser$_{136,}$ iNOS, eNOS or cleaved caspase-3 activity as described below.

2.7 QUANTITATIVE ANALYSIS OF p-BAD $_{(Ser136)}$, T-BAD, iNOS, GAPDH, p-eNOS $_{(Ser\ 1177)}$ AND eNOS $_{(Ser\ 1177)}$ BY FACS ANALYSIS

Following the different treatments myocytes were harvested and centrifuged at 1200 rpm for 2 minutes. The supernatant discarded while the pellet was resuspended in Phosphate Buffer Saline (PBS) and fixed with 3% formaldehyde for 10 minutes at room temperature. The fixed cells were then put on ice for 1 minute before centrifuging at 1200rpm for 2 minutes and the supernatant discarded. 250 µl of ice cold methanol (90%) was added and the samples incubated on ice for 30 minutes before being washed twice in incubation buffer (0.5% BSA in PBS) following a 10 minutes (at 37°C) incubation of the samples each time followed by centrifugation of the samples (at 1200rpm, 2 minutes).

FACS was used to assess the differential protein levels. For p-BAD $_{(ser136)}$ and T-BAD analysis, the samples were probed for 1 hour with either p-BAD $_{(ser136)}$ or T-BAD rabbit monoclonal antibody diluted at 1:100 dilution in incubation buffer followed by incubation in Alexa Fluor® 488 goat anti-rabbit IgG antibody at a dilution of 1:1000 for 1 hour. For iNOS and GAPDH, the samples were also probed for 1 hour with iNOS or GAPDH rabbit monoclonal antibody diluted at 1:100 dilution in incubation buffer followed by incubation in Alexa Fluor® 488 goat anti-rabbit IgG antibody at a dilution of 1:1000 for 1 hour. For p-eNOS and e-NOS the samples were also probed for 1 hour with p-eNOS $_{(Ser\ 1177)}$ and eNOS $_{(Ser\ 1177)}$ rabbit monoclonal antibody diluted at 1:100 dilution in incubation buffer followed by incubation in Alexa Fluor® 488 goat anti-rabbit IgG antibody at a dilution of 1:1000 for 1 hour.

At the end of the incubation period, the cells were centrifuged and the supernatant was removed the cells resuspended in 500µl PBS and analysed using flow cytometer (Becton Dickinson, Oxford, UK) on the FL1 channel to count 10,000 events (Vermes *et al.*, 2002; Hussain *et al.*, 2014).

2.8 QUANTITATIVE ANALYSIS OF CLEAVED CASPASE-3 ACTIVITY

Cleaved caspase-3 (Asp175) Antibody (Alexa Fluor 488 conjugate) purchased from New England Biolabs (Ipswich, UK) was used to detect the cleaved caspase-3 a pivitol effector caspase in apoptotic signalling (Sakamaki and Satou 2009). Activation of caspase-3 requires

proteolytic processing of its inactive zymogen into p17 and p12 fragments. The antibody detects endogenous levels of large fragments of cleaved caspase-3 (Sakamaki and Satou 2009). At the end of the experimental protocol the cells were harvested from a 24 well plate and transferred to a labelled 1.5 ml microfuge tubes containing treated cells and controls were centrifuged at 1200 rpm for 2 minutes. The pellet was then resuspended in Phosphate Buffer Saline (PBS) and fixed with 3% formaldehyde for 10 minutes at room temperature.

The cells were then fixed on ice for 1 minute before centrifuging at 1200 rpm for 2 minutes following aspiration of the supernatant. 250 µl of ice cold methanol (90%) was added and the samples incubated on ice for 30 minutes. Myocytes were subsequently washed with incubation buffer twice (0.5% BSA in PBS) and blocked with incubation buffer for 10 minutes and spun again at 1200 rpm for 2 minutes (at 37°C). The cells were then fixed for 10 minutes at 37ºC and put on ice for 1 minute before centrifuging at 1200 rpm for 2 minutes following aspiration of the supernatant. 250 µl of ice cold methanol (90%) was added and the samples incubated on ice for 30 minutes before being washed twice in incubation buffer (0.5% BSA in PBS) following a 10 minutes (at 37°C) incubation of the samples each time followed by centrifugation of the samples (at 1200rpm, 2 minutes). The antibody was prepared to 1:100 final dilution in incubation buffer. The cells were then incubated in incubation in Alexa Fluor® 488 goat anti-rabbit IgG antibody at a dilution of 1:1000 for 1 hour in the dark at room temperature. The cells were then spun at 1200 rpm for 2 minutes and washed with incubation buffer twice. At the end of the incubation period, the cells were centrifuged and the supernatant discarded. The pellet was then resuspended in 500µl PBS and analysed using flow cytometer (Vermes *et al.*, 2002). Samples were then analysed using the flow cytometer (FACS, Becton Dickinson, Oxford, UK) on FL-1 channel and set up to count to 10,000 events.

2.9 CELLULAR VIABILITY ASSAY BASED ON MTT REDUCTASE ACTIVITY USING ISOLATED VENTRICULAR CARDIOMYOCYTES

After 2 hours of hypoxia/reoxygenation respectively, the cells were subjected to MTT assay to measure succinate dehydrogenase activity. This assay measures the ability of NADPH-dependent cellular oxidoreductase enzymes within viable cells to reduce MTT tetrazolium dye from yellow to purple (as shown in Figure 12, Figure 13). Cells were placed into a 96 well flat-bottomed microtitre plate with 50 µl of cells (containing 1×10^{-4} cells.ml^{-1}) per well. 10 wells were used as control and contained 100µl of restoration buffer. Other wells were used for the

different treatment groups which contained 50µl of cells and 50µl of drug treatment. Drugs used for this study were diluted with restoration buffer to a final concentration of 4.3µM in the artemisinin treated group, 0.1µM in wortmannin treated groups, 0.1µM in rapamycin treated groups, 100µM in L-NAME treated group and 100µM in aminoguanidine treated group.

The total number of cells was calculated in order to determine the volume of RB to resuspend the cells in and obtain 10,000 cells per ml, this was determined using a nucleo counter (Chemometec, Sartorius, and Surrey, UK). 1ml of cells was stored for normoxic control and 1ml for hypoxia/reoxygenation control. 6 wells were used for each concentration of the experimental groups used. Cells were then incubated for 2 hours under hypoxic conditions. Drugs were administered at the start of reoxygenation and cells were incubated for an additional 2 hours with 20µl of MTT (MTT solution consisting of $5mg.ml^{-1}$ in PBS (10g for 10^{-4} cells/well) was added at reoxygenation except for the blanks which contains restoration buffer and MTT solution. Cells were incubated in the dark at 37^0C for 2 hours. Upon completing the 2hours reoxygenation, myocytes were subsequently lysed with 100µl of lysis buffer (20% SDS in 50% dimethylformamide) and incubated on an orbital shaker and incubated overnight at 37^o C. Calorimetric analysis of the plate was done to measure the fluorescence emission at 450nm (Thermo Scientific, UK) using a plate reader (Anthos 2001).

Figure 12*. The principle behind the formation of formazan from NADPH-dependent cellular oxidoreductase enzymes within viable cells which reduces MTT tetrazolium dye from yellow to purple. Adapted from (NBSBio, 2010).*

Figure 13. *MTT assay showing a yellow tetrazole in the first two columns (control) which was reduced to purple formazan showing the proportion of living cells in the wells. Adapted from* *http://www.nature.com/nprot/journal/v3/n3/images/nprot.2007.517-F1.jpg*

The absorbance was measured for the different treatment groups. The effect of the artemisinin treatment was obtained by subtracting the absorbance from the control values. Graphs were made using the mean absorbance of the drug treated group as a percentage of the mean absorbance of the control group.

2.10 CYTOTOXICITY ASSAY BASED ON MTT REDUCTASE ACTIVITY USING HL-60 CELL LINE

Human leukaemia cancer cell line HL-60 was obtained from the European Collection of Cell Cultures (ECACC). The cells were cultured in RPMI 1640 media without L-Glutamine (Biosera, Ringmer, UK) which was supplemented with 10% fetal bovine serum, 2mM L-glutamine, HEPES and 0.1% antibiotic solution (100 U/ml penicillin, 0.1mg/ml streptomycin; Invitrogen Paisley,UK). The cells were maintained at 37°C under a humidified atmosphere and 5% CO_2. Cell viability was measured using an electronic counter (NucleoCounter ®) and culture plastics purchased from Thermo Scientific (Roskilde, Denmark).

In the MTT (3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) assay a yellow tetrazole was reduced to purple formazan which was proportional to the amount of living cells, thus determining the number of viable cells incubated per plate. Incubation was at 37°C and its duration was based on results of assay optimization experiments from previous experiments in

the lab (data not presented). HL-60 cells ($1x10^4$cells/well) were plated in 96-well flat-bottomed microtitre plates, and treated with varying concentrations of Artemisinin (10-1000µM), Artesunate (0-60µM) and Dihydroartemisinin (0-60µM) for 24 hours. At the end of the incubation period, the cells were terminated by adding MTT solution (20µl of 5mg/ml MTT to each well) and further incubated for 2 hours at 37°C. 100µl of lysis buffer (20% sodium dodecyl sulfate in 50% dimethyl formamide) was then added to each well containing treatment and control samples.

The plates were incubated overnight to solubilise the cells. The absorbing intensity of each well was determined at 492nm using a plate reader (Anthos 2001). This measures the percentage of cell growth calculated using the following formula;

% Cell Growth = (average absorbance of treated wells for Artemisinin/average absorbance of untreated control wells) x 100%.

The experiment was repeated three times and IC_{50} values for artemisinin was estimated using the 4-parametric logistic analysis (Grafit Software, Erithacus, UK). The mean calculated IC_{50} value was then used in subsequent experiments in the chapters to follow.

Dose–response titration was plotted for artemisinin, artesunate dihydroartemisinin to yield concentrations of half-maximal inhibition (IC_{50}).

Using MTT assay in cancer cells is a widely accepted method of measuring cell proliferation. It determines the number of live cells per well as a percentage of control thus allowing accurate representation of drug exposure to cancer cells. Despite its limitation it is a rapid and cheap way of assessing cellular proliferation calometrically and it is proven to be valid, accurate and easily reproducible method of analysis.

Detection sensitivity however varies widely among different cell types, depending on the metabolic activity of the cell type being tested. Typically HL-60 cells proliferate quickly and are easy to culture. Due to these reasons and the widely available validation of MTT assay using HL-60 cancer cells by many researchers, this study chose to use HL-60 cells.

2.11 NITRIC OXIDE ASSAY USING THE OXISELECT™ IN VITRO NITRIC OXIDE CALORIMETRIC ASSAY

Nitric Oxide is an important mediator in the pathogenesis of several diseases (Isenovic *et al*., 2011; Raij 2006). Reactive nitrogen intermediates (RNI) and reactive oxygen intermediates (ROI) especially, have been shown to damage cells by inactivating the metabolic enzymes and damaging important cellular components which exacerbate diseases such as ischaemia, atherosclerosis, inflammatory diseases and cancer (Rahman *et al*., 2012; Isenovic *et al*., 2011). At the moment, the concept of oxidative stress does not include 'nitrosative stess' which includes RNI such as nitric oxide (NO), peroxynitrite and, recently, to S-nitrosothiols which have been shown to react with proteins, carbohydrates and lipids, with consequent alterations to intracellular and intercellular homeostasis, leading to possible cell death and regeneration (Rahman *et al*.,2012).

Figure 14. *Schematic representation of nitric oxide and nitric oxide intermediates and their effects on normal cellular homeostasis to cell death. Adapted from <http://file.scirp.org/Html/8-7300384/9f214aba-c126-45e5-874d-3721fd603aa4.jpg>*

Due to the extremely short half-life and difficulty in quantification of NO, total NO is measured as the sum of the oxidized nitrate and nitrite (Bryan and Grisham 2007) which offers valuable

information regarding NO bioavailability and metabolism. Nitrate in the sample is converted to nitrite by nitrate reductase enzyme. The OxiSelect™ quantitatively measures this using the Greiss reagents, a coloured azo dye which absorbs at 540nm.

2.11.1 Experimental protocol using the Nitric Oxide assay

Adult ventricular rat myocytes were isolated from Sprague Dawley rats by enzymatic dissociation method as in the previous protocols. Myocytes were counted using a haemocytometer and resuspended in restoration buffer (RB) to a density of 100,000 cells/ml.

2.11.2 Preparation of Samples

Samples are filtered through with 10kDa MWCO ultrafilter to reduce protein interference and turbidity.

2.11.3 Preparation of standards

Reagents are prepared according to manufacturer's instructions. With unused stock aliquoted and frozen to avoid multiple freeze thaw cycles.

Nitrite and nitrate standards were prepared by making a dilution series of concentration range 0-140µM from a standard 14mM of the nitrite and nitrate provided in the kit in restoration buffer. Potential interference was prevented by diluting the nitrate and nitrite standard in the same buffer as the samples, although this can compromise sensitivity.

2.11.4 Assay Protocol

Nitrate levels in the samples were measured by subtracting nitrite only from total nitrite and nitrate.

Nitrate= Total nitrite + nitrate -Nitrite only

2.11.5 Measurement of Nitrite only

- 50µL of nitrite standards, samples or blanks were added to a 96 well plate
- 50µL of PBS was added to each of the wells
- 50µL of Griess Reagent A was added to each well
- Plate was incubated at 10 minutes, this allowed the colour to develop and absorbance was read at 540nm on a microplate reader

Concentration of nitrite was calculated by comparing sample absorbance to standards

Negative Control without nitrate were subtracted

Each nitrite standard and sample was assayed in duplicate

2.11.6 Measurement of Total/Nitrate via Nitrate Reduction

- 50µL of nitrate standard, samples or blanks to the 96 well plate
- Enzyme reaction mixture was prepared for the number of tests to be performed
- Appropriate volume of diluted enzyme cofactor is added (1:100 dilution in deionized water) and vortexed
- 50µL of the enzyme reaction mixture is added to the wells containing the sample/nitrate and covered with foil
- Plates are then incubated for 1 hour at room temperature on an orbital shaker
- 50µL of Greiss Reagent A is added and vortexed followed by Gneiss Reagent B
- Plate is incubated for a further 10 minutes for colour development
- Absorbance is then read at 540nm

The sample's absorbance is then calculated by comparing the Nitrate and standard curve.

2.12 WESTERN BLOTTING

2.12.1 Introduction to Western blotting

Western blotting, also called immunoblotting is a commonly used analytic technique used to detect identify and quantify fractioned proteins based on its molecular weight, charge and conformation. The protein is extracted from an extract of tissue or cell homogenate. Proteins are separated based on the size by gel electrophoresis and transferred unto a PVDF membrane by immunoblot transfer (Mahmood and Yang 2012). The proteins then adhere to the membrane in the same pattern as they have been separated due to interactions of charges. They are then probed with specific primary and secondary antibody of the protein of interest. The membrane is then developed for the visualization of specific protein bands to measure the relative density of each protein. The size of the tagged protein is detected by imaging which then determines the accurate molecular weight of the target protein. The molecular weight of which corresponds to a band on the marker weight marker used. However the effectiveness of this technique can be limited to high levels of nonspecific background staining which can limit the identification and/or quantification of band intensity (Olle *et al.*, 2005).

2.12.2 Tissue Preparation

At the end of the Langendorff experiment, tissues were harvested for western blot analysis. The hearts collected underwent 20 minutes stabilisation, 35 minutes ischaemia and varying

reperfusion times (10 minutes, 20 minutes, 60 minutes and 120 minutes) in the presence and absence of artemisinin and the inhibitors. This reperfusion times are based on established protocols used in our lab, with 10 minutes reperfusion sample ran in the cell signalling studies. Upon completing the experimental protocol, the hearts were removed from the Langendorff apparatus and the left ventricles were cut off using a sterile scalpel which was then snap frozen in liquid nitrogen and stored at -80°C for further analysis.

2.12.3 Protein Extraction

Proteins were extracted from the stored frozen samples. Approximately 60mg of the frozen ventricular tissue sample was placed in a sterile cryogenic vial containing 250 µl of cold suspension buffer (100 mM NaCl, 10 mM Tris, 1 mM EDTA, (pH 8.0), 2 mM Sodium pyrophosphate, 2 mM sodium fluoride, 2 mM β-glycerophospahte, 0.1 mg/ml PMSF, Roche Complete™ protease tablet followed by high speed centrifugation using IKA Labortechnik T25 homogeniser at 11,000rpm at 4^{o}C for 10minutes). The supernatant were assessed for protein concentration using the Nanodrop spectrophotometer ND1000 (Thermo Scientific, UK) at 280nM. The samples were then diluted with an equal volume of sample buffer (312mM Tris-Cl pH6.8, 50% Glycerol, 0.15% Bromophenol Blue, 2.5% SDS) followed by heating for 5mins at 95^{o}C and centrifuging for 30secs at 5000rpm. The remaining of the undiluted protein samples were stored at -80°C as stock protein concentrations.

2.12.4 Gel electrophoresis

60 µg of protein sample was loaded unto Biorad 4-15% Tris/Glycine precast gradient gels (Biorad, Hertfordshire, UK). The gel was then attached to the Mini-PROTEAN 3 electrode assembly system (Biorad PowerPac 3000) with the short plates facing inwards and run at 130 volts for 90 minutes. The electrode assembly was then placed into the clamping frame and securely closed. The inner chamber is then lowered into the Mini Tank which was placed in the Biorad mini protean III system (Bio-Rad, UK) which separates out the samples.

Approximately 125 ml of running buffer (glycine 14.42 g/l, SDS 1.0 g/l, Tris 3.0 g/l) was added to the inner chamber of the system and about 200 ml added to the outer chamber. The plastic combs were removed to expose the wells and samples loaded into the appropriate wells using gel loading tips (Fischer). A protein molecular marker (Cell Signalling Technologies, UK) with bands of specific molecular weight was also loaded to one of the wells for identification of the target protein. The gel was then run by attaching electrical leads from the Mini-PROTEAN 3 apparatus to the Power-Pac using the Power-Pac 3000 at 130 volts for 90 minutes.

2.12.5 Protein transfer

The gels were then transferred to a Polyvinyl Di fluoride (PVDF) membrane using the Trans-Blot Turbo transfer packs (Bio-Rad, UK) assembled in accordance to the manufacturer's guidelines. Turbo transfer mixed molecular weight transfer settings were selected on the transfer system to transfer at 25V, 1.3 A for 7 minutes when using two gels.

2.12.6 Immunoblot Transfer Procedure

The PVDF membrane was washed in Tris-buffered Saline Tween 20 (TBST) and incubated with blocking buffer (5% milk in (TBS) Tween 20 TBST) for an hour facing upwards. The membrane was washed 3 times in TBST and then incubated overnight on an orbital shaker at 4^{o}C with the primary antibody phospho-Akt$_{Ser473}$ or phospho-p70S6K $_{(Thr\ 389)}$ rabbit monoclonal antibody at a dilution of 1:1000 for 12-14 hours.

After the incubation, the membrane was washed 3 times in TBST and then incubated in a 1:2000 dilution of Anti-rabbit antibody HRP linked IgG and HRP linked anti-biotin antibody orbital shaker at room temperature for 1 hour. The membrane was washed again 3 times in TBST before imaging.

Upon capturing the images of the relative changes in density, the blots were then stripped of the phospho-antibody (p-Akt $_{(Ser\ 473)}$ or p-p70S6k $_{(Thr\ 389)}$) by boiling in water for 5 minutes and membranes re-probed for T-Akt or T-p70S6K by incubating in (5% milk in TBST) for an hour, then incubated overnight on an orbital shaker at 4^{o}C for Totals (T-Akt and T-p70s6k) at a dilution of 1:1000. After the overnight incubation, the membrane was washed 3 times in TBST and then incubated in a 1:2000 dilution of Anti-rabbit antibody HRP linked IgG and HRP linked anti-biotin antibody. Immunoblots were detected on the Biorad™ ChemiDoc imaging system as done earlier.

2.12.7 Quantification of band density using Chemiluminescent Phospho-imagery

The immunoblots were used to detect the proteins by using the enhanced chemiluminescence substrates. Membranes were placed on a piece of acetate sheet with 1 ml of Super Signal West Femto (Fischer Sceintific, Loughborough,UK) which was prepared by combining reagent A and B in 1:1 dilution and was added on top of the membrane prior to use while keeping it away from light. The solution was spread over the entire surface of the membrane with excess substrate dripped off. The membrane was then placed into the using the Biorad™ ChemiDoc imaging system (Bio-Rad, UK). Using the Bio-Rad Quantity One software the membrane is exposed for 10-30 seconds allowing the proteins bands to be visualised. Band density was

analysed by taking into account background exposure for the blot using Image J (N.I.H, Bethesda, USA) and accurately identifying the target protein.

2.13 miRNA PROTOCOL

2.13.1 Evaluation of miRNA profile of the isolated perfused heart tissue following Artemisinin treatment

2.13.1.1 qPCR for the determination of miRNA expression in the myocardium.

Hearts were subjected to the different treatments as described in the isolated perfused rat heart model with the exception of inserting the latex balloon for haemodynamics readings. Following the Langendorff perfusion, the left ventricle was dissected and chopped into smaller pieces and transferred into 2ml RNase/DNase free microfuge tubes containing 500ml of RNA later solution (Ambion life technologies, Paisley, UK) which was then stored in a – 20 ºC freezer until use.

Quantitative reverse transcriptase PCR was used to analyse the expression of cardiac injury specific miRNAs (miR-1, miR-27a, miR-133a and miR-133b) and a miRNA involved in cancer development miR-155.

2.13.1.2 miRNA extraction from isolated ventricular tissue following Artemisinin treatment in the isolated perfused heart model

miRNA was extracted using the Mirvana™ miRNA Isolation kit (Ambion, Applied Biosystems, UK) in accordance with the manufacturer´s instructions. The homogeniser (IKA Overtechnical T25) was cleaned with with with RNase/DNase free ethanol (Fischer, UK), ZAP solution and RNase free H20 (Ambion Life Technologies Paisley, UK). The RNA later solution in which the sliced ventricles were placed was discarded and 1 ml of lysis buffer (Ambion Life Technologies Paisley, UK) solution was added to the tissue which was placed in ice. The samples were then homogenised using the IKA Overtechnical T25 in ice, making sure that the samples remained cool to prevent denaturation of the RNAs by over homogenising. 30 µl of miRNA homogenate additive (Ambion Life Technologies Paisley, UK) was added to the tissue lysate, vortexed and incubated on ice for 10 min.

Using the extraction hood, 300µl of chloroform (Ambion, UK) was added to the microfuge tube containing the tissue lysate. The samples were then vortexed and miRNA Homogenate Additive was added, vortexed again and centrifuged at 12,000 rpm at room temperature. The upper phase (containing RNA) was transferred to new RNAase free microfuge tubes while the lower phase (containing chloroform and phenol) was carefully discarded. The sample was subsequently spun for 5 min at 10,000g using (mikro 200R Hettich Zentrifuge). Some RNAase free H_2O is heated on a heating block at 95 ºC for elution. The upper phase which has been transferred into a new RNAase free tube (while noting the volume transferred each time for the different sample).

1.25 x volumes of 100% RNAase free ethanol (EtOH) was added to the aqueous phase collected (ie. to 500 µl of upper phase collected; 625 µl of EtOH was added). The mixtures were centrifuged through a spin column into a collection tube for 15 seconds at 12,000 rpm at room temperature. Maximum volume filtered at a time was 700 µl so the process was repeated until all of the mixture is filtered. The effluent was discarded and the spin column was washed with 500 µl of Wash Solution 1 (Ambion, UK) which was filtered through the filter cartridge and then centrifuged for 15 seconds at 12,000rpm at room temperature. The flow through was discarded. Next, 500µl of wash solution 2/3 was added to the spin column and centrifuged for 15 seconds at 12,000 rpm at room temperature. The last wash was repeated at the same speed. The filter was then spun with the emptied collection tube for 1 min at 12,000 rpm to remove any residual fluid and a new filter placed in a newly marked collection tube.

50 µl of 95 °C warm RNase free water (Ambion, UK) was added to this new spin column and incubated for 1 min. The spin column/ collection tubes were centrifuged for 20 seconds at 12,000rpm, at room temperature. The flow through contained the extracted miRNA which was collected and stored in – 20 ºC freezer.

2.13.2 Quality control

The quantity and quality of RNA obtained was measured using the nanochip bioanalyser (Quiagen) and nanodrop-1000 spectrophotometry (NanoDrop Technology, Delaware, USA).
Nanochip Bioanalyser (Qiagen).

The nanochip bioanalyser (Qiagen) was used to assess RNA quality, 1µl of RNA (~250ng) was used. The RNA electrodes were thoroughly cleaned with RNA RNAZap (Ambion, UK) and

Deionised water. The samples to be run and the RNA 6000 Ladder were heated to 70°C for 2 minutes to thaw and placed on ice until use. The filtered gel matrix was then prepared by adding 550µl of RNA matrix into a spin filter and centrifuged at 1500 × *g* for 10 minutes. 65µl of the eluate and 1µl of RNA dye is then added to the RNAase-free tubes and placed on ice which was then centrifuged at 13,000 × *g* for 10 minutes. 9µl of this gel dye mix was used to prepare the RNA 6000 nanochip in the chip priming station (which is located on well C4). Upon priming the chip, an extra 9µl was then added into each well of the chip marked with a G (that is well A4 and B4). 5µl of RNA 6000 Nano Marker was then placed into the remaining 13 wells of the chip with1µl of each sample and 1µl of RNA 6000 Ladder added to the appropriate wells on the chip. The chip was then vortexed for 1 minute and used for the *Eukaryotic total RNA Nano* assay.

2.13.3 Nanodrop-1000 spectrophotometry

Nanodrop-1000 spectrophotometry (NanoDrop Technology, Delaware, USA). Was used at an absorbance of 280 nM to determine the RNA integrity number (RIN). This ensures high purity levels from the extracted RNA. The eluate which contained the RNA was collected and stored at -20°C for RT-PCR and qPCR.

2.13.4 Reverse transcription reaction

Quantitative polymerase chain reaction, also called quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR) or RT-PCR. A widely used technique used to detect, amplify and quantify specific sequences in a DNA molecule. The technique involves conversion of RNA into complementary DNA (cDNA) using reverse transcriptase. The cDNA is then used as a template for exponential amplification using PCR. For each cycle run, RNA levels are duplicated. The amplified RNA can then be quantified using the computer software.

To convert RNA template into a complementary DNA (cDNA), 500 ng miRNA was reverse transcribed for U6 snRNA, rno-miR-1, hsa-miR-27a, hsa-miR-133a, hsa-miR-133b and hsa-miR-155 using the Applied Biosystems MicroRNA Reverse Transcription Kit and primer assay set (Applied Biosystems, USA) according to the manufacturer's instructions. miRNA expression patterns were assessed by reverse transcription MicroRNA Assays (Applied Bio systems) followed by QP using the different stem-loop primers.

DNA/RNA free microfuge PCR tubes were labelled and the following was added:

For each RT reaction 0.3µl of 100 mM dNTPs, 1.5µl of MultiScibe™ Reverse Transcriptase, 3µl of 10x RNase buffer, 0.3µl of RNase Inhibitor and 0.5µl of the different TaqMan miRNA primers x 6 = 3µl TaqMan RT PCR miRNA primer mix (making a total of 8.1µl of RT PCR Master mix master mix) . A total volume of 30µl for the reaction, 21.9µl being nuclease water which was added to each well.

For each RT reaction below were the reagents used;

		1x reaction	**10x reaction**
1	100 mM dNTPs	0.3µl	3.00 µl
2	Multi Rev Trans	1.5µl	15.00 µl
3	10x RT buffer	3.00 µl	30.00 µl
4	RNAse inhibitor	0.3 µl	3.00 µl
5	U6	0.50 µl	5.00µl
6	miR-1	0.50 µl	5.00 µl
7	miR-27a	0.50 µl	5.00µl
8	miR-133a	0.50 µl	5.00 µl
9	miR-133b	0.50 µl	5.00µl
	Total vol master mix	8.1µl	

Table 1. *Concentration of reagents used in RT Reaction*

- RNAase free H_2O: To make Vt uptill 30 µl - 8.1 µl= 21.9 µl

- Each sample will have a different miRNA concentration and therefore will need a specific volume of miRNA and water to each sample RT PCR reaction

The 6 primer sets used are: U6, miR-1, miR-27a, miR-133a, miR-133b and miR-155, all from Ambion life sciences (Paisely, UK).

To convert RNA template into a complementary DNA (cDNA), Quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR) was performed with the following setup; 16 °C for 30 minutes, 42 °C for 30 minutes and 85 °C for 5 minutes. This exponentially amplifies the RNA with duplication of RNA with each cycle.

2.13.5 Real-Time PCR reaction

miRNAs expression patterns were assessed by reverse transcription with TaqMan MicroRNA Assays (Applied Biosystems) followed by real time PCR..

Real Time PCR was performed using a standard real time PCR protocol on the 7500 HT Real Time PCR sequence detection system (Applied Biosystems, USA). The 7500 Fast Real Time PCR sequence detection software SDS software version 1.4 (Applied Biosystems, UK) was used to record the amplification of DNA in real time by optics and imagining system using SYBR Green fluorescent dye incorporation to double stranded DNA. A 20µl reaction mixture containing 100 ng cDNA, specific Applied Biosystems miRNA primer assays and SYBR green PCR Master Mix was used in the Real Time PCR reaction. A non-template control was included in all experiments.

For the Real Time PCR reaction 0.5µl of each primer set (Applied Viosystems primer assays) 10µl 2xSYBR green PCR Master Mix (SYBR MM) and 6.5µl nuclease free water making a total of 17µl volume of the non-template controls with 3.0µl of cDNA for the experimental group. Each 20 µl reaction contained the following:

cDNA	3 µl
Individual Primer set	0.5 µl
SYBR MM	10 µl
H_2O	6.5 µl

***Table 2**: The volumes of reactions needed for real time PCR reactions*

The order is adding the reagents to reduce tip wastage are:

a) RNase free H_2O

b) Master mix "mix" (Primers and SYBR MM)

c) cDNA

A typical layout used for the qPCR plate design for the reaction is as in the following table:

	1	2	3	4	5	6	7	8	9	10	11	12
A	U6	U6	miR-1	miR-1	miR-27a	miR-27a	miR-133a	miR-133a	miR-133b	miR-133b	miR-155	miR-155
B	U6	U6	miR-1	miR-1	miR-27a	miR-27a	miR-133a	miR-133a	miR-133b	miR-133b	miR-155	miR-155
C	U6	U6	miR-1	miR-1	miR-27a	miR-27a	miR-133a	miR-133a	miR-133b	miR-133b	miR-155	miR-155
D	U6	U6	miR-1	miR-1	miR-27a	miR-27a	miR-133a	miR-133a	miR-133b	miR-133b	miR-155	miR-155
E	U6	U6	miR-1	miR-1	miR-27a	miR-27a	miR-133a	miR-133a	miR-133b	miR-133b	miR-155	miR-155
F	U6	U6	miR-1	miR-1	miR-27a	miR-27a	miR-133a	miR-133a	miR-133b	miR-133b	miR-155	miR-155
G	U6	U6	miR-1	miR-1	miR-27a	miR-27a	miR-133a	miR-133a	miR-133b	miR-133b	miR-155	miR-155
H	NTC	NTC	NTC	NTC	NTC	NTC	NTC	NTC	NTC	NTC	NTC	NTC

Real Time PCR reaction was performed using the following setting; 50 °C for 2 minutes, 95 °C for 10 minutes, followed by 40 cycles of 95 °C for 15 seconds and 60 °C for 1 minute.

Programme:

1) 2 min 50 ºC

2) 10 min 95 ºC

3) 15 sec 95 ºC

4) 1 min 60 ºC

5) repeat 3) and 4) 40 times

2.13.6 Quantifying miRNA results:

The ΔΔ cycle threshold (CT) values for the artemisinin treated group which was then compared with the control group. U6 was used as an internal reference to normalise other primers.

2.13.6.1 Statistical analysis:

Fold changes in the miRNA expressions were assessed for statistical differences using ANOVA (GraphPad Prism version 5 and SPSS). All values were expressed as mean ± SEM. P-values of ≤0.05 were considered statistically significant. The relative amount of miRNA was calculated with the CT values for the different primers in relation to the CT values of U6 snRNA using the following formula; miRNA data was then analysed using the comparative ΔΔCT method (Sandhu, 2010).

$X_0/R_0 = 2^{CTR-CTX}$

Where;

X_0 = original amount of target miRNA

R_0 = original amount of U6 snRNA

CTR = CT value for U6 snRNA, and

CTX = CT value for the target miRNA.

2.14 STATISTICAL ANALYSIS ACROSS TECHNIQUES USED:

The percentage of infarct/risk ratio, % of cell viability, cleaved caspase-3 levels, eNOS (Ser 1177), iNOS, the relative changes in phosphorylated proteins and miRNA expressions were all expressed as MEAN±SEM. Hearts treated with artemisinin, the different inhibitors and the control groups were tested for group differences in infarct size, cellular viability and protein expression using the SPSS software package-One Way analysis of variance (Anova) with LSD post hoc test. Physiological parameters (Haemodynamics) were assessed using Two Way Anova. P values of $P<0.05$ were considered statistically significant.

Chapter 3

3 PROFILING ARTEMISININ: ROLE OF ARTEMISININ IN MYOCARDIAL ISCHAEMIA-REPERFUSION/ HYPOXIA-REOXYGENATION

3.1 INTRODUCTION

Artemisinin is described as a sesquiterpene trioxane lactones used as anti-pyretic treatment for fever and flu in ancient China (Ho *et al.*, 2014). Artemisinin is used especially in malaria treatment which is converted in the body to the active metabolite, dihydroartemisinin (DHA) (Zhu *et al.,* 1983 and Melendez *et al*., 1991).

Artemisinin has a wide range of biological functions ranging from anti-malarial, anti-tumour, anti-microbial and anti-inflammatory activities (Bilia *et al.,* 2014 and Lai *et al.,* 2005). Malaria remains one of the major threats to human health, in developing countries especially affecting early two billion people are at risk all over the world and an estimated one million die of the disease annually (Wang *et al*., 2010).

It is however well established that antimalarial drugs especially the quinines and quinolones present associated neurotoxicity and cardiotoxicity in experimental models (Balint, 2001; Kinoshita *et al*., 2010; Hara *et al.,* 2007). Several cardiotoxic effects such as impaired left ventricular contractility, heart failure and increased risk of death (Lipshultz *et al*., 2004 and Kinoshita *et al*., 2010) have been associated with the different antimalarial treatments such as halofantrine. Although very effective in treating multidrug resistant *P falciparum,* Ter Kuile *et al*. (1993) reported the first death related to halofantrine cardiotoxicity. Another patient case study evaluation revealed 74% of fatal events occurring within 24 hours of administering halofantrine (Bouchard *et al*., 2009; Ter Kuile *et al*., 1993). The use of antimalarial drugs for therapeutic reasons was thus limited due to the drug resistance and associated neurotoxicity and cardiotoxicity observed across the different treatments.

However, aside from its current use as an anti-malarial drug, the artemisinin's (artemisinin and its derivatives) have also shown a potential in treating several infections such as trypanosomiasis and leishmania, inhibit several viruses, such as human cytomegalovirus and other members of the Herpes viridae family (herpes simplex virus type 1 and Epstein-Barr virus), cancers and inflammation (Efferth *et al*., 2008; Mishina *et al*., 2007; We *et al*., 2014). All these findings made the artemisinin's interesting and important to review especially as a potential anti-cancer therapy.

Recent research has shown the artemisinin's express cytotoxic effects against a variety of cancer cells by inducing cell cycle arrest at various stages of the cell division depending on the cancer cell line (Yoon *et al.*, 2012) by promoting apoptosis and preventing tissue invasion, angiogenesis and metastasis (Ho *et al*., 2014; Crespo-Ortiz and Wei, 2011; We *et al.*, 2014).

The adverse cardiotoxic effects presented by the popular antimalarial drugs are also common amongst traditional cancer therapy drugs (Dudgeon *et al*., 2002 Although quite efficient in killing cancer cells often exarcerbate co-morbid effects in patients suffering from heart disease (Daniels *et al.*, 2012).

Anti-cancer therapy has seen much development over the years, resulting in improvement of life expectancy across patients of different cancer types (Ibrahim *et al*., 2013). However, with this advancement, patients are now surviving long enough for the prevalence of adverse cardiovascular effects posed by some of the cancer therapies to become evident (Bowles *et al*., 2012; Chen *et al*., 2012; Yeh *et al*., 2004). This poses a need for the development of effective treatments that reduce drug induced cardiotoxicity and protect the myocardium from injury damage (Broder *et al*., 2008).

Cancer cells contain high levels of required iron for proliferation; however this makes it an ideal target for artemisinin by causing the release of ROS which has been shown to trigger apoptosis via oxidative stress (Efferth 2005; Crespo-Ortiz and Wei, 2011). A study using HL-60 cell lines have shown artemisinin induced damage is related to early and rapid generation of ROS which was found to activate apoptosis (Michaelis *et al*., 2010).

ROS induced stress has been shown to increase mitochondrial oxidative stress (Cui *et al.*, 2012 and Dai *et al*., 2014). As mentioned in chapter 1, increase in ROS in tissues as a result of reperfusion injury can damage macromolecules within the tissue which eventually causes myocardial cell death (Banerjee *et al*., 2008; Palcher *et al.*, 2007). This process is shown to be

primarily mitochondrial mediated causing abnormal increase in calcium and several other cellular deregulations (Wang 2001). The complex roles of the mitochondria in apoptosis involve several mitochondrial proteins which activate different apoptotic signals that leads to mitochondrial swelling (Du *et al.,* 2000; Verhagen *et al*., 2000). Mitochondria swelling can cause the outer mitochondrial membrane to rupture and release cytochrome c into the cytosol forms complexes known as apoptosomes (Montaigne e*t al*., 2012). Apoptosomes comprise of procaspase-9 which are cleaved to caspase-9 which activates caspase-3 promoting apoptosis (Yuan *et al*., 2011). There is now evidence showing, artesunate a derivative of artemisinin activates mitochondrial apoptosis in breast cancer cells via iron catalysed lysosomal ROS generation (Brady *et al*., 2010). Apoptosis being a tightly regulated cell deletion process, plays an important role in various cardiovascular diseases, such as myocardial infarction, reperfusion injury andother coronary diseases leading to heart failure.

The irreversible damage to cardiac tissue caused as a result of myocardial ischaemia reperfusion injury sustained during ischaemia and reperfusion has been shown to activate apoptosis although the exact time at which this occurs is unclear (Kalogeris *et al.,* 2012; Palcher *et al*., 2007). Studies have however confirmed that in addition to apoptosis caused as a result of ischaemic injury, increase in apoptosis is observed with reperfusion treatment. This is due to additional stress inflicted on the already weakened cells, to rapidly recover after being severely compromised during ischaemia (Zhoa *et al.,* 2002). Studies have shown the elderly population are more prone to developing ischaemic heart disease as well as other diseases due to the decrease in function of cells as a result of aging (Stern *et al.,* 2003; Simanek *et al.,* 2011). Substantial evidence has also shown that the presence of comorbidities such as ischaemic heart disease in diseases such as cancer compromises therapeutic advances in oncology (Tashakkor *et al.,* 2013). The prevalence of comorbidities is particularly high in the elderly population, with 80% of this population having three or more chronic conditions at a time (Caughey *et al.,* 2008). In this review by Caughey *et al.* (2008), comorbidities have been associated with a decline in many health outcomes as well as with an increase in mortality rates. A significant number of studies have also identified the correlation between the incidences of cancer and increase in age, while reporting improvements in life expectancy as a result of medical and pharmacological interventions (Crivellari *et al.,* 2000; Simanek *et al.,* 2011). More cancer patients are reported to survive cancer upon treatment however these cancer survivors are identified to be at greater risks of developing cardiovascular diseases which may act as a potential hazard when providing appropriate treatment to these cancer patients (Tashakkor *et*

al., 2013). Studies have reported this population of cancer survivors are more likely to develop secondary malignancies such as ishaemic heart disease partly because of their age but more significantly as a result of drug induced cardiotoxity from the anthracycline therapy (Gharanei *et al.*, 2013, Tashakkor *et al.*, 2013). Presently administered cancer treatment such as doxorubicin which is a first line treatment against a wide range of cancers including haematological malignancies, solid tumours and soft sarcomas (Tacar *et al*., 2013). Although doxorubicin is extremely effective in treating these cancer types, its use is limites as it has been shown to exacerbate myocardial injury particularly in patients with underlying ischaemic heart disease, this has been reported as a threefold increase in the rate of cardiomyopathy in the elderly population (Doyel *et al.*, 2005). This population of patients with pre-existing conditions/comorbidities are very often under represented in studies. With the possibility of both conditions (ishaemic heart disease and cancer) to co-exist at the same time in patients undergoing cancer treatment it is imperative to study both conditions at a cellular level in terms of apoptosis which may lead to death.

Lee and Gustafsson (2009) have implicated apoptosis in the pathogenesis of a variety of cardiovascular diseases and also reported that the inhibition of apoptosis is cardioprotective and can prevent the development of heart failure. With this knowledge the inhibition of cardiac apoptosis therefore holds promise as an effective therapeutic strategy for cardiovascular diseases (King and Kang 2010).

Advancements have been made in the clinical development using miRNA as therapeutic targets and biomarkers of cardiovascular diseases (Olson 2014). miRNA's are small non coding RNA molecules, found to freely circulate in plasma and are responsible for regulating gene expression by recognizing complementary messengers (Dimmeler *et al.*, 2010; Corsten *et al*., 2010). miRNAs play important regulatory roles in homeostasis and disease not just in the cardiovascular system (Corsten *et al*., 2010). Many studies have explored the diagnostic potential in a selection of miRNA's and have identified peculiar miRNA expression profiles that correlate with a variety of pathological conditions such as hypertrophy, heart failure, and arrhythmias (Schroen *et al*., 2009; van Rooij *et al.*, 2006). miRNA found freely circulating in blood are easily detected suggesting the potential of being useful disease biomarkers (Dimmeler *et al.*, 2010). In the cardiovascular system, miRNA's are generally considered to act as intracellular mediators required for maintaining normal cardiac function (Wang and Yang 2012). Deregulation in the expression profiles of the miRNA molecules are associated with

cardiovascular diseases such as pathological cardiac hypertrophy and heart failure in humans and mouse models of heart diseases (Salic and Windt 2012; Wang and Yang 2012).

miRNA-1 and miRNA-133 is the most abundantly expressed miRNA in the heart (Basser *et al*., 2014). D'Alessandra *et al*. (2010) showed that circulating muscle-derived miRNAs might be useful biomarkers of acute myocardial infarction. They have also been found to regulate various processes ranging from normal physiological conditions to stressed and to even diseased conditions thus rendering them as potentially novel biomarkers for disease onset (Schlutz *et al.*, 2014; Kondkar and Abu-Amero 2015). Recent studies have identified circulating microRNAs as possible novel biomarkers that may be used in the diagnosis of acute myocardial infarction (Li *et al.,* 2013). Prior to miRNAs B-type natriuretic peptide (BNP) and cardiac troponin have been thoroughly used in studies investigating heart failure and acute myocardial infarction (Jiang *et al.,* 2014). BNP is a useful biomarker in emergency setting with serum level associated with age, race and body mass index whereas cardiac troponin is a prognostic indicator of acute MI where serum level is shown to elevate with conditions such and hypertension and ketoacidosis (Li *et al.,* 2013). However, more recent have suggested miRNAs to be more sensitive and reliable biomarkers in sub-clinical myocardial injury (Li and Zhang 2015). Li *et al*. (2014) investigated the expression of plasma miRNA-1 and cardiac troponin T in early diagnosis of patients with acute myocardial infarction where they showed that miRNA is more specific and sensitive in early diagnosis compared to cardiac troponin T however it is not superior.

miRNAs can potentially be used for the early detection and identification of diseases (Zhoa *et al.,* 2010; Jiang *et al*., 2014). With vascular diseases being prevalent worldwide, early assessment and ability of miRNAs to clearly show the stages of diseases alone or in association with previous biomarkers such as BNP, have emerged with more sensitive and increased diagnostic powers (Jiang *et al.,* 2014).

Studies have further suggested that miRNA-1 and miRNA-133 for example which is released into blood during cardiac tissue injury is upregulated in response to tissue injury otherwise associated with pro-apoptotic effects (Tang *et al.,* 2009; Ho *et al*., 2011). miRNA-1 in adult cardiomyocytes and skeletal muscle is known to regulate apoptosis, by targeting the synthesis of (heat shock protein-60) HSP-60, (heat shock protein-70) HSP-70, and Bcl-2 (B cell lymphoma-2, an apoptosis regulator) (Yang *et al*., 2007). HSPs are a family of proteins produced in response to stress (Yang *et al*., 2007). Some genes are aberrantly expressed in

infarcted hearts, miRNA's such as miRNA-1 which is consistently dysregulated in ischaemic hearts and miRNA-27a which is highly expresses in breast cancer cells (Tang *et al*., 2009; Wu *et al*., 2011; Mertens-Talcott *et al.,* 2007). Researchers found that overexpression of these miRNA'S could trigger increased sensitivity to I/R injury or even trigger cell death or in the case of miRNA-27a its suppression decreases the number of breast cancer cells through the inhibition of cell cycle traverse and increased apoptosis (Wu *et al*., 2011;Liu *et al.,* 2009). In this study we therefore detected the expression profile of popular miRNA's associated with ischaemia reperfusion or cancer in naïve and artemisinin treated cardiomyocytes.

At present there is renewed interest in developing anti-cancer therapies from medicinal herbs which have been found to be generally safer than first line therapies currently being administered in treatment. The artemisinin's stand out as a family of bioactive molecules with high potency against several cancer cells (Ho *et al.,* 2012). Studies have shown the artemisinin's offer a promising alternative due to their established safety record in anti-malarial treatment and the promising effect against 55 different cancer cell lines including breast cancer, leukaemia, prostate, ovarian and so on (Efferth *et al.,* 2003; Lai *et al*., 2013; Tin *et al*., 2012; Chadwick *et al.,* 2010; Zhou *et al.,* 2008; Crespo-Ortiz and Wei 2011). These studies emphasised the important role of artemisinin as an effective and possibly potent anticancer treatment with much promise. Coupled with the findings by Sun *et al.,* (2007) who showed artemisinin at high micromolar concentrations to be cardioprotective against myocardial ischaemia/reperfusion (I/R) injury in rats and further suggested the mechanism may be related to its functions of antioxidation and scavenging free radicals.

For decades now, it is well established that most of the widely used cancer limited in use due to their dose dependent cardiotoxicity (Harake *et al*., 2012). As previous limited studies have revealed the potential of artemisinin to be cardioprotective against myocardial I/R injury (Sun *et al.,* 2007). However no study is yet to establish the intracellular signalling via which this occurs. The cardiovascular system is undoubtedly a complex well organised system where signal transduction plays an important role in its normally physiology as well as pathophysiology (Wheeler-Jones 2005). It is therefore imperative to investigate the artemisinin mediated cardioprotection suggested by Sun *et al.* (2007) and in subsequent chapters we investigated the intracellular signalling associated with the cardioprotection, as no study has established that to our knowledge. Furthermore, previous studies have revealed the importance

of prosurvival kinases especially Akt, in hearts recovering from ischaemia reperfusion injury (Whittington *et al.*, 2013).

Aims of the study: In this chapter however, we aimed to establish the effect of artemisinin in naïve isolated perfused hearts as well as in stressed conditions and in isolated cardiomyocyte subjected to I/R and H/R respectively. We furthermore, investigated the differential expression of certain miRNA upon treatment with artemisinin as well as investigating artemisinin's cytotoxicity against HL-60 cancer cells.

3.2 METHODOLOGY

3.2.1 Materials

(3*R*,5a*S*,6*R*,8a*S*,9*R*,12*S*,12a*R*)-Octahydro-3,6,9-trimethyl-3,12-epoxy-12*H*-pyrano[4,3-*j*]-, 2-benzodioxepin-10(3*H*)-one (Artemisinin) purchased from Tocris (Bristol, UK), which is dissolved in dimethyl sulfoxide (DMSO) and stored at -20 °C. Thiazolyl blue tetrazolium bromide (MTT) was purchased from Sigma (Poole, UK). Cleaved Caspase-3 (Asp175) Antibody (Alexa Fluor® 488 Conjugate) purchased from New England Biolabs (Hertfordshire, UK). *mir*Vana™ miRNA Isolation kit equipments and primers used for miRNA Isolation were all purchased from Applied Biosystems, UK (Ambion, Applied Biosystems, UK),Applied Biosystems MicroRNA Reverse Transcription Kit and primer assay set for U6 snRNA, rno-miR-1, hsa-miR-27a, hsa-miR-133a, hsa-miR-133b and hsa-miR-155 (Applied Biosystems, UK), TaqMan MicroRNA Assays (Applied Biosystems, UK) and TaqMan Universal PCR Master Mix purchased from (Applied Biosystems, UK). Human Leukamia cells (HL-60) were obtained from the European Collection of Cell Cultures (ECACC)

3.2.2 Animals

Adult male Sprague-Dawley rats 350-400g were obtained from Charles River (Margate, UK) for this study. Animals received humane care in accordance with the Guidance on the Operation of the Animals (Scientific Procedures Act 1986) and were sacrificed by cervical dislocation.
The study was carried out upon obtaining ethical approval from Coventry University Research ethics committee which was regularly assessed throughout the project.

3.2.3 Isolated perfused rat heart model

Briefly following sacrifice, the hearts were rapidly excised and placed on a langendorff set-up where it is retrogradely perfused with KH buffer as described in detail in chapter 2.

The pH of the KH buffer was maintained at 7.4 by gassing continuously with 95% O_2 and 5% CO_2 maintained at a temperature of 37 ± 0.5ºC using a water-jacketed heat exchange coil.

A latex balloon inserted into the left atrium which is inflated to constant diastolic pressure of 8-10mmHg in the left ventricle is used to measure left ventricular developed pressure (LVDP). A physiological pressure transducer was connected to a bridge amp and a power lab (AD Instruments Ltd, Chalgrove, UK) allowing the LVDP, HR (HR) and CF (CF) was measured at regular intervals while collecting the perfusate at regular intervals. Hearts maintained a steady state of LVDP, HR and CF.

The langendorff experiment was conducted for 175 minutes in total. In the normoxic experiments, the isolated hearts were perfused for 155 minutes with KH buffer after 20 minutes stabilisation.

Hearts were randomly assigned to the following different treatment groups. In the normoxic treatment groups:

a) hearts perfused with KH buffer alone (Normoxic control) for 155 minutes after 20 minutes stabilisation.
b) hearts perfused with KH buffer and Artemisinin (Normoxic drug treatment; 10nM-100µM) (Artemisinin was administered throughout 155 minutes of perfusion)

In the Ischaemia/reperfusion (I/R) studies, hearts were allowed to stabilise for 20 minutes followed by 35 minutes ischaemia and 120 minutes reperfusion. The anterior descending left coronary artery was ligated to induce regional ischaemia. Upon completing the 35 minutes of simulated ischaemia, the flow of KH buffer was reintroduced for 120 minutes (Figure 10).

In the Ischaemic control group, after 20 minutes of stabilisation, ischaemia was induced for 35 minutes and drug treatment administered during reperfusion for 120 minutes. Hearts were also randomly assigned to the following different treatment groups;

a) hearts that underwent I/R were perfused with KH buffer alone (I/R control)
b) hearts that underwent I/R were perfused with KH buffer and artemisinin treatment (I/R drug treatment; artemisinin (10nM-100µM) was administered throughout reperfusion).

At the end of the experiment, the hearts were incubated in triphenyl tetrazolium chloride (TTC) solution (1% in phosphate buffer) for 10–12 minutes and fixed in 10% formalin for at least 4

hours to enhance the staining prior to analysis. The dye stains any risk tissue bright red and infarct tissue pale/whitish or was frozen in RNA later (Applied Bio systems, UK) for miRNA analysis.

The heart slices were then treaced unto acetate film and scanned into a computer to allow calculation of I/R% as areas of viable, risk and infarct tissue measured using the Image Tool program as developed by the University of Texas Health Science Centre at San Antonio, Version 8.1 (UTHSCSA).

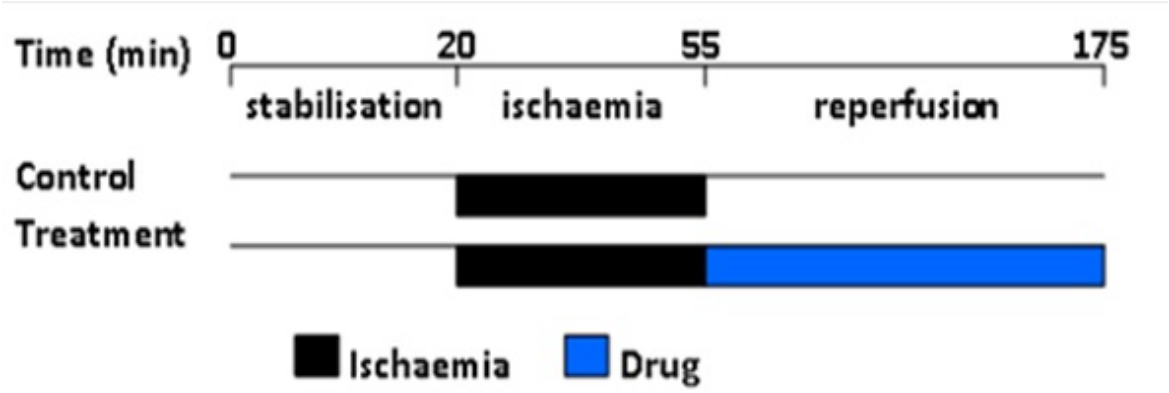


Figure 15. *Protocol for Infarct/risk ratio analysis and tissue collection for miRNA analysis*

3.2.4 Evaluation of miRNA profile in isolated perfused heart tissue following drug treatment

50 mg of heart tissue was snap frozen in snap frozen in RNA*Later,* (after subjected to the different treatments as described in the isolated perfused rat heart model). miRNA was extracted using the Mirvana™ miRNA Isolation kit (Ambion, Applied Biosystems, UK) in accordance with the manufacturer´s instructions.

cDNA was then made by reverse transcribing 500 ng miRNA for U6 snRNA, rno-miR-1, hsa-miR-27a, hsa-miR-133a, hsa-miR-133b and hsa-miR-155 using the Applied Biosystems MicroRNA Reverse Transcription Kit and primer assay set (Applied Biosystems, UK) according to the manufacturer's instructions.

miRNAs expression patterns were assessed by reverse transcription TaqManR MicroRNA Assays (Applied Biosystems) followed by qPCR using the different stem-loop primers.

Real Time PCR was performed using a standard TaqMan Universal PCR Master Mix (Applied Biosystems, USA) protocol on the 7500 HT Real Time PCR sequence detection system (Applied Biosystems, USA). The SDS software version 1.4 was used to record the

amplification of DNA using SYBR Green fluorescent dye to double-stranded DNA (Applied Biosystems, UK).

Upon completing the Real Time PCR reaction the ΔΔ cycle threshold (CT) values for the Artemisinin treated group was compared with the control group. U6 was used as a reference to normalise other primers.

Fold changes in the miRNA expressions were assessed for statistical differences using the Students t-test (GraphPad Prism version 5). All values were expressed as mean ±SEM. A P-value of $P \leq 0.05$ was considered statistically significant. miRNA data was then analysed using the comparative ΔΔCT method (Sandhu, 2010).

The relative amount of miRNAs were calculated with the CT values for the different primers in relation to the CT values of U6 snRNA using the following formula;

$$X_0/R_0 = 2^{CTR-CTX}$$

Where;

X_0 = original amount of target miRNA

R_0 = original amount of U6 snRNA

CTR = CT value for U6 snRNA, and

CTX = CT value for the target miRNA.

3.2.5 Adult Rat Ventricular Myocytes Model

Adult ventricular rat myocytes were isolated from Sprague Dawley rats (350-400g) by enzymatic dissociation method as described in chapter 2. The isolated myocytes were incubated in RB (at 37 ^{o}C, 5% CO_2 for 24 hours before being used.

Control sample was pulled aside for the normoxic group. The rest of the myocytes then underwent reoxygenation for 2 hours. Upon completing reoxygenation, the isolated cardiomyocyte underwent quantitative analysis using Fluorescence Activated Cell Sorter (FACS) to assess cleaved caspase-3 activity, iNOS or eNOS $_{(Ser1177)}$ expression or using MTT reductase (Thiazolyl blue tetrazolium bromide) for assessment of cellular viability.or flow cytometric analysis for the assessment of cleaved caspase-3 activity as described below.

3.2.6 Assessment of cleaved-caspase 3 activity by FACS

Isolated cells were harvested, washe and incubated in inubation buffer as described in chapter 2. The antibody was prepared to 1:100 final dilution in incubation buffer. The cells were then incubated in incubation in Alexa Fluor® 488 goat anti-rabbit IgG antibody at a dilution of 1:1000 for 1 hour in the dark at room temperature. The cells were then spun at 1200 rpm for 2 minutes and washed with incubation buffer twice. At the end of the incubation period, the cells were centrifuged and the supernatant discarded. The pellet was then resuspended in 500µl PBS and analysed using flow cytometer (Vermes *et al*., 2002). Samples were then analysed using the flow cytometer (FACS, Becton Dickinson, Oxford, UK) on FL-1 channel and set up to count to 10,000 events. Histograms were then plotted to record the levels of cleaved Caspase-3 activity. Please refer to methods chapter 2 for detailed description of the technique.

3.2.7 MTT cell viability assay using isolated cardiomyocytes:

Following 2 hours of hypoxia, cardiac myocytes were harvested and subjected to MTT assay to measure MTT reductase activity. This assay reduces the initial yellow tetrazole to purple formazan, which quantifies the proportion of viable cells incubated per plate. Cell densities were initially calculated to 100,000 cells/ml. The cells were then placed into a 96 well plate with 100 µl of cells (1×10^{-5} cells.ml^{-1}) per well. Normoxic control and Hypoxia reoxygenated untreated cells were isolated while the remaining myocytes were treated with artemisinin (4.3µM) throughout the 2 hours of reoxygenation. 20µl of MTT (MTT solution is 5mg.ml^{-1} in PBS (10g for 10^{-4} cells/well) was added to all the wells and incubated in the dark at 37^0C for 2 hours. 100µl of lysis buffer (20% SDS in 50% dimethylformamide) was then added and incubated overnight at 37º C. Calorimetric analysis of the plate was done to measure the fluorescence emission at 450nm on the NanoDrop 2000c UV-Vis Spectrophotometer (Thermo Scientific, UK).

3.2.8 MTT cell viability assay using HL-60 cell line:

Human leukaemia cancer cell lines HL-60 were obtained from the European Collection of Cell Cultures (ECACC). The cells were cultured in RPMI 1640 media without L-Glutamine (Biosera, Ringmer, UK) and supplemented with 10% fetal bovine serum, 2mM L-glutamine, HEPES and 0.1% antibiotic solution (100 U/ml penicillin, 0.1mg/ml streptomycin from Invitrogen). The cells were maintained at 37°C under a humidified atmosphere and 5% CO_2. Cell viability was measured using an electronic counter (NucleoCounter ®). HL-60 cells were counted on the nucleocounter, 50µL of the cells containing ($1x10^5$cells/well) was plated in 96-

well flat-bottomed microtitre plates and treated with varying concentrations of artemisinin (10-1000µM) for 24 hours. At the end of the incubation period, the cells were terminated by adding MTT solution (20µl of 5mg/ml MTT to each well) and further incubated for 2 hours at 37°C. 100µl of lysis buffer (20% sodium dodecyl sulfate in 50% dimethyl formamide) was then added to each well containing treatment and control samples.

Incubation was at 37°C and its duration was based on results of assay optimization experiments from previous experiments in the lab (data not presented in this study). The plates were incubated overnight to solubilise the cells. The absorbing intensity of each well was determined at 492nm using a plate reader (Anthos 2001). This measures the percentage of cell growth calculated using the following formula;

% Cell Growth = (average absorbance of treated wells for artemisinin/average absorbance of untreated control wells) x 100%.

The experiment was repeated three times and IC_{50} values for artemisinin were estimated using the 4-parametric logistic analysis (Grafit Software, Erithacus, UK). The mean calculated IC_{50} value was then used in subsequent experiments in the chapters to follow.

Dose–response titration was plotted for artemisinin (1-1000µM) to yield concentrations of half-maximal inhibition (IC_{50}).

Statistical Analysis

The data obtained was expressed as % of infarct/risk ratio, % of area at risk/ventricular volume, % of cell viability, % cleaved caspase-3 levels, the relative quantitative changes in phosphorylated proteinssuch as iNOS and eNOS and miRNA expressions were all expressed as MEAN±SEM. The different groups were tested for group differences using the SPSS software package - One Way analysis of variance (ANOVA) with LSD post hoc test. Physiological parameters (haemodynamics) were also assessed using a one way ANOVA for each time point. Fold changes in miRNA study were assessed for statistical difference using student t-test. P values of $P<0.05$ were considered statistically significant.

3.3 RESULTS

3.3.1 Exclusion Criteria

For this chapter two hearts were excluded from this experiment, one was due to poor stabilization at the beginning of the experiment and the other due to very high flow rate which may be indicative of a tear in the aortic wall or may be an incompetent valve. Another rat was also excluded due to low viability of live cells following isolation (≤70%) in the cellular viability studies. All other groups have been used.

3.3.2 Profiling the effects of artemisinin treatment on haemodynamics in isolated perfused hearts under normoxic conditions

Normoxic hearts were allowed to stabilise for 20 minutes followed by perfusion with KH buffer for 155 minutes. Artemisinin treated hearts were stabilised for 20 minutes followed by artemisinin treatment with $EC_{80:}$ 4.3µM for 155 minutes (Figure 2). 4.3µM is the dose calculated using I/R heart where the cardioprotective effects were more significant compared to the untreated control group (Figure 20b and 20c) (where 80% drug response is seen).

Left ventricular developed pressure (LVDP), Heart rate (HR) and CF (CF) haemodynamic parameters were assessed throughout the time period of the experiments detailed above.

The artemisinin (4.3µM) treated normoxic hearts remained relatively stable in terms of LVDP when compared to the normoxic control hearts throughout reperfusion. The artemisinin (4.3µM) treatment alone did not appear to have a significant effect on LVDP when compared to control but the artemisinin (4.3µM) treatment was slightly higher, although not significantly different from the control in LVDP when perfused with artemisinin (4.3µM) for 155 minutes, as illustrated in Figure 16.

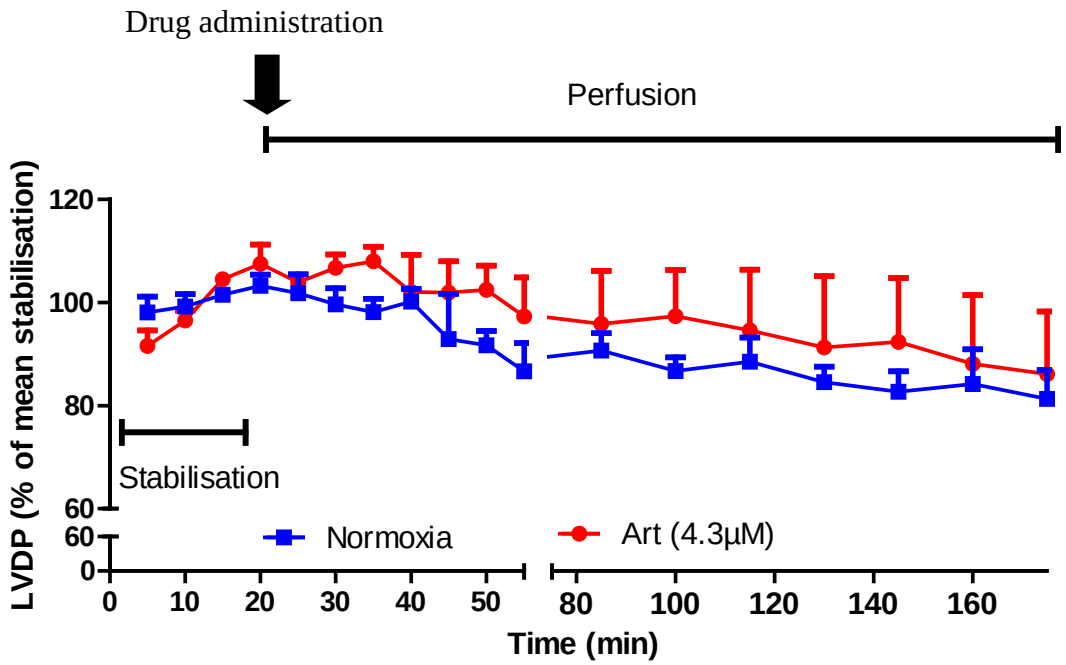


***Figure 16**. The effects of artemisinin (4.3µM) on LVDP in isolated perfused hearts under normoxic conditions expressed as a percentage of mean stabilisation. Hearts were subjected to 20 minutes of stabilisation and were perfused for 155 minutes in the presence/absence of artemisinin (4.3µM). Results presented are Mean±SEM (n=6).*

3.3.2.1 The effects of artemisinin (4.3µM) treatment on HR in isolated perfused hearts under normoxic conditions

Artemisinin (4.3µM) treated group, compared to time matched normoxic control showed a progressive increase in HR ($P<0.001$). Artemisinin (4.3µM) treatment caused a significant increase in the HR, this is noticeable 10 minutes into treatment with artemisinin (4.3µM) (97.2±2.7% vs. 111.6±9.7%, $P<0.05$, Figure 17). The effect became more pronounced at the later stages of the treatment when compared with the control group (at 125 minutes: 91.0 ± 1.9% vs. 115.6 ± 10.8% respectively, $P<0.001$, as seen in the following Figure 17.

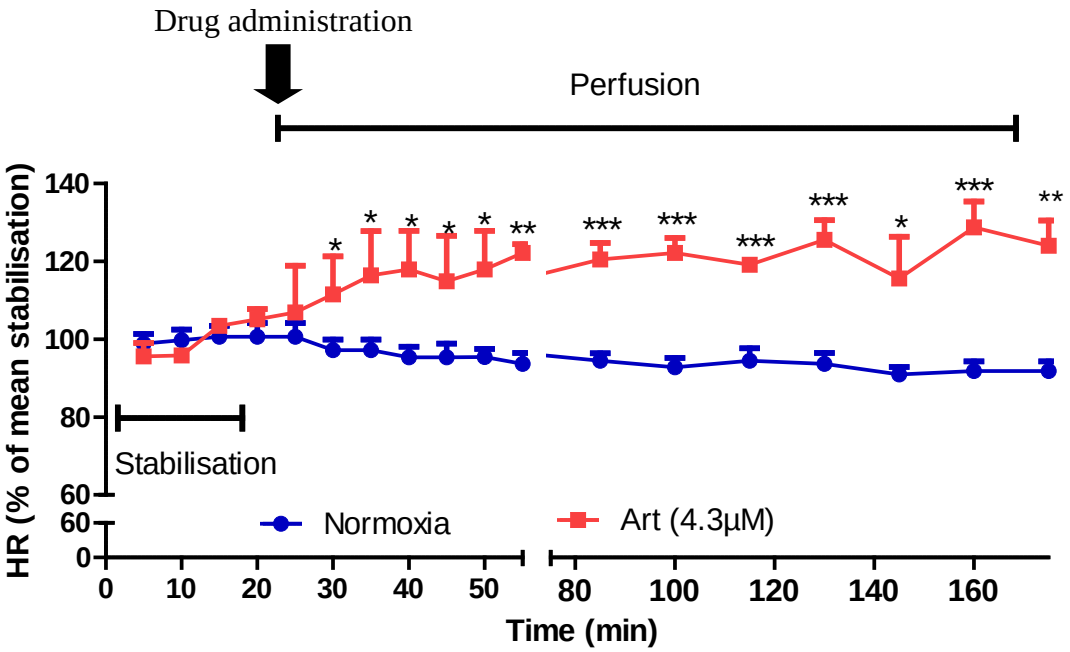


Figure 17. *The effects of artemisinin (4.3µM) on HR in isolated perfused hearts under normoxic conditions expressed as a percentage of mean stabilisation. Hearts were subjected to 20 minutes of stabilisation and were perfused for 155 minutes in the presence/absence of artemisinin (4.3µM). Results presented are Mean±SEM (P<0.05 vs. Normoxic Control, **P<0.01 vs. Normoxic Control, ***P<0.001 vs. Normoxic control) (n= 6).*

3.3.2.2 The effects of artemisinin (4.3µM) treatment on CF in isolated perfused hearts under normoxic conditions

CF was also recorded by collecting the effluent at regular intervals of 5 minutes for 1 minute. CF in the artemisinin treated group compared to the normoxic time matched control showed a very similar pattern, however artemisinin (4.3µM) treatment led to a significant (P<0.05) increase in CF at 5 minutes and 10 minutes of perfusion with artemisinin (4.3µM) (92.1±4.3% vs. 104.7±3.5% in artemisinin treated hearts at 5 minutes of drug perfusion) and (93.8±2.5% vs.103.8±4.0% in artemisinin treated hearts at 10 minutes of drug perfusion). Most significant increase in CF was observed at 170 minutes (i.e. 120 minutes into the treatment with artemisinin (4.3µM) when compared with untreated normoxic control (at 155 minute of drug perfusion; 88.3 ± 2.7% vs. 97.6± 2.2%, P<0.01, Figure 18).

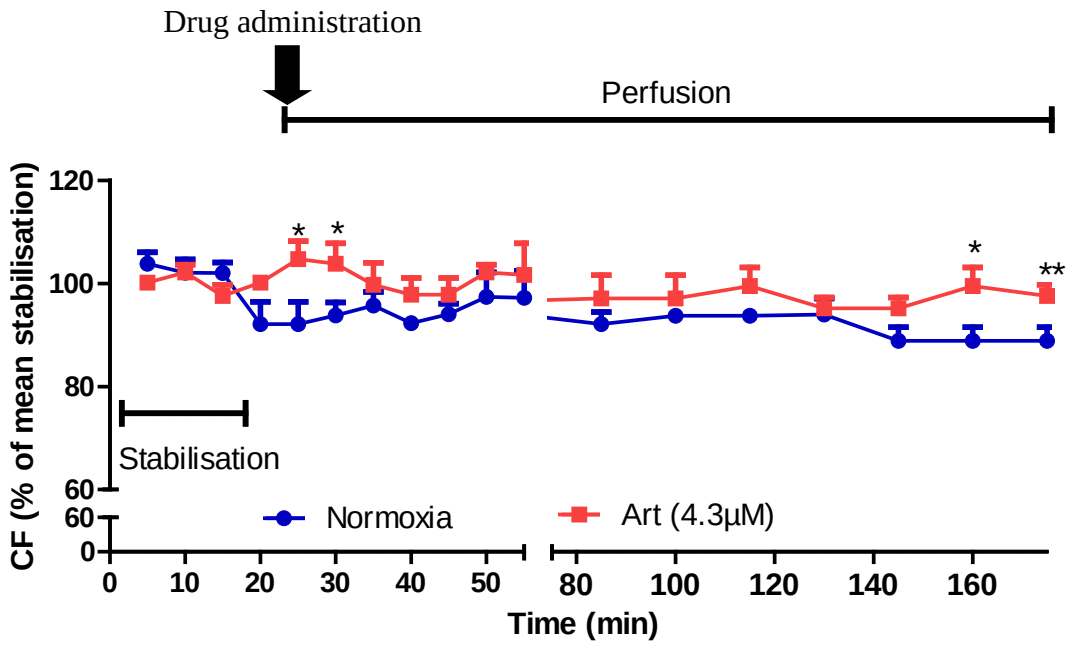


Figure 18. *The effects of artemisinin (4.3µM) on CF in isolated perfused hearts under normoxic conditions expressed as a percentage of mean stabilisation. Hearts were subjected to 20 minutes of stabilisation and were perfused for 155 minutes in the presence/absence of artemisinin (4.3µM). Results presented are Mean±SEM (n=6)*

3.3.3 Effects of artemisinin treatment on infarct size in isolated perfused hearts in normoxic conditions

Infarct size was calculated as a percentage of the area at risk. This is reported to be the most vital, reliable and reproducible way of assessing infract size and has been used by several studies in understanding mechanisms behind these common diseases, and also towards developing and evaluating the most appropriate treatment strategies (Redford *et al.,* 2012). Different hearts are different in size and in terms of haemodynamics and electrophysiology of the heart. Diverse morphological differences therefore may exist between the hearts sampled particularly when measuring infarct size may, this may be a crucial limitation of this technique. However by measuring infarct size as a percentage of the area at risk, (area at risk being the area that correlates with the entire myocardial perfusion bed distal to the occluded coronary artery) calculating infarct size thus accounts for variability within the groups in terms of dissimilarities in the actual size of the heart and size of the area occluded (ischaemia induced) (Graham *et al.,* 2001). This is the most accepted way of determining the actual infarct size and may be used for prognosis and evaluation of drug efficacy especially in studies aimed at reducing infarct size or in studying genetic manipulation on the ischaemic tolerance of the myocardium (Graham *et al.,* 2001; Liu *et al*., 2002).

TTC is used to assess infarction based on the percentage of area at risk, this is an effective way of investigating diseased conditions (Redford *et al.*, 2012).

The area at risk is thus a potential source of variation in infarct analysis so this study quantified it to eliminate possible variabilities and also calculated the significant difference between the experimental groups.

Treatment with artemisinin (4.3µM) for 155 minutes was compared to untreated time matched control hearts. Administration of artemisinin (4.3µM) significantly decreased the infarct size when compared to the untreated control (8.93±1.16% vs. 6.58±0.23%, P<0.05, Figure 18)

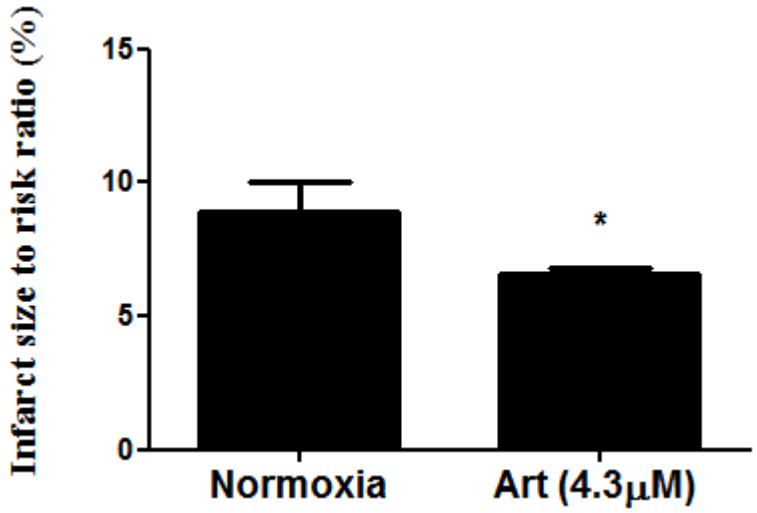


Figure 19. *Infarct to risk ratios in the isolated rat hearts subjected to 20 minutes of stabilisation followed by 155 minutes perfusion with KH buffer in the presence/absence of artemisinin (4.3µM). Results were expressed as Mean±SEM (*P<0.05 vs. normoxic control) (n=6)*

3.3.3.1 The effect of artemisinin on isolated perfused hearts subjected to I/R injury

The cardioprotective effect of the increasing concentrations of artemisinin (0-100µM) was investigated in order to establish the concentration at which artemisinin is most protective while monitoring the effect on its different effects on the haemodynamic parameters observed as well as on infarct size.

3.3.3.2 The effect of artemisinin (0-100µM) treatment on (%) area at risk/ventricular volume in isolated perfused rat hearts subjected to I/R injury.

All the hearts in this group were allowed to stabilise for 20 minute while perfused with KH buffer, hearts where then subjected to 35 minutes ischaemia followed by 120 minutes reperfusion with artemisinin (0-100µM). At the end of the reperfusion period, the left coronary artery was re-occluded and stained with Evans blue which was injected into the left ventricle

to stain the ischaemic area and risk area. The Evans blue stains the non-ischaemic tissue blue while the ischaemic area/ infarcted region becomes pale. The area at risk (ischaemic area) was quantified for each heart to ensure the values do not vary significantly between the groups (Figure 20a). This allows for better assessment and quantification of the % of infarcted tissues as well as for more reliable quantification of drug treatment. Quantifying infarct size as a percentage of area of risk between the groups corrects for variability in heart sizes and induced ischaemia between the different hearts and aso the groups. Administering artemisinin at 0.01µM concentration compared with I/R showed no significant difference (42.0± 4.6% vs. 37.9±3.1% respectively, Figure 20a). The % area at risk/left ventricular volume for the increasing concentrations of artemisinin also showed no significant difference to control and between the groups (Figure 20a).

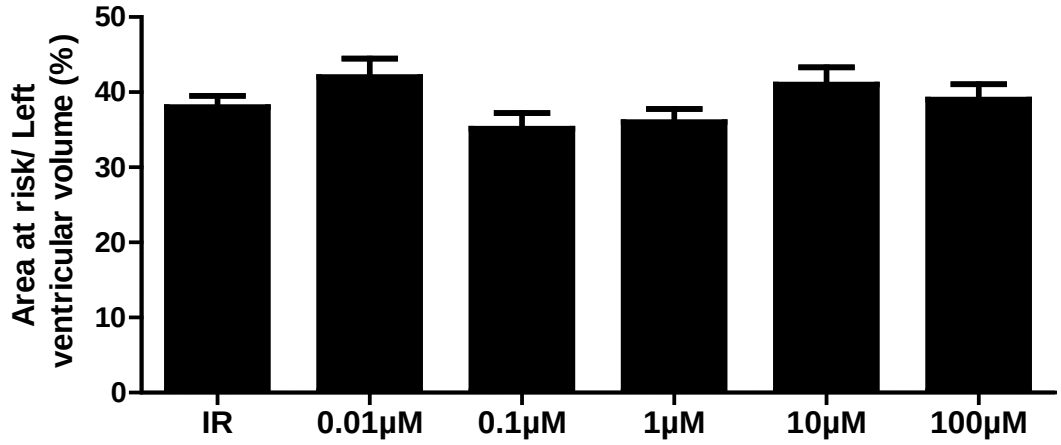


Figure 20a*. Area at risk expresses as a percentage of the left ventricular volume. Hearts were subjected to 20 minutes stabilisation, 35 minutes ischaemia and 120 minutes reperfusion in the presence/absence of artemisinin (0-100µM). Results were expressed as Mean±SEM*

3.3.3.3 The effect of artemisinin (0-100µM) treatment on Infarct size to Risk ratio (%) in isolated perfused rat hearts subjected to I/R injury

For the infarct size analysis, hearts were stained with TTC. The hearts were treated with artemisinin over a wide range of significantly lower concentration's (0-100µM) than previously tested by Sun *et al.*, 2007. Upon reperfusion at 0.1µM, artemisinin started showing a significant infarct sparing effect when compared to untreated time matched I/R control (46.9±1.5% vs. 55.8±1.7% I/R, $P<0.01$,Figure 20b). There was a gradual decrease in infarct size with increasing concentration of artemisinin, with results being highly significant compared to control. With an increased concentration of artemisinin of up to about 10µM, infarct size significanty decreased by up to 20% compared to I/R control (35.7±3.5% vs. 55.8±1.7% I/R,

P<0.001, Figure 20a). Artemisinin (0.1µM-100µM) showed a significant dose dependent decrease in infarct size (P<0.01-0.001 vs. I/R control) when compared to untreated time matched I/R control.

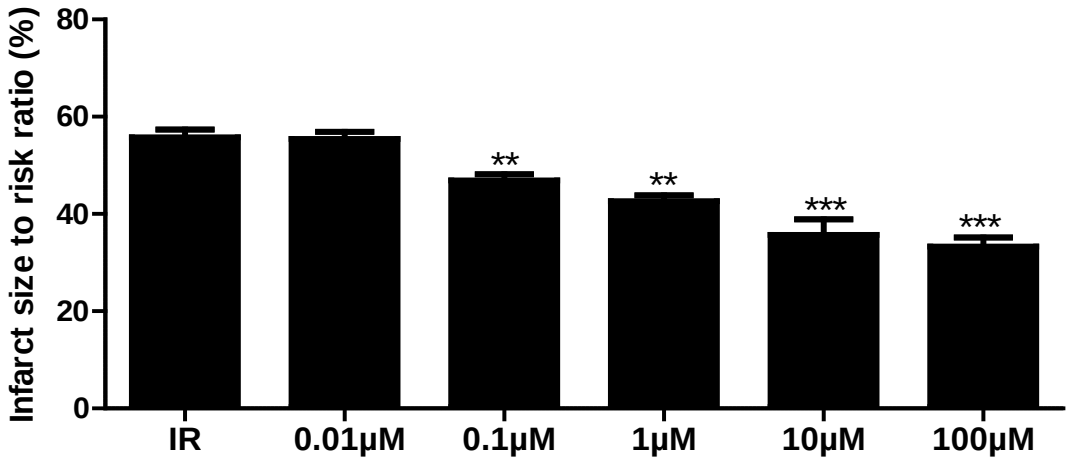


Figure 20b. *The effects of artemisinin (0-100µM) on infarct size to risk ratios in isolated perfused rat heart model of I/R. Hearts were subjected to 20 minutes stabilisation, 35 minutes ischaemia and 120 minutes reperfusion in the presence/absence of artemisinin (0-100µM). Results were expressed as Mean±SEM (**P<0.01 vs. I/R control, ***P<0.001 vs. I/R control) (n=4-8) (Artemisinin=Art)*

The infarct sizes were used to construct a dose response curve and the EC_{20}, EC_{50} and EC_{80} values were calculated with these values used for the subsequent experiments. EC_{20} value was calculated as 0.0042µM using the values from the concentrations above and as shown in the log graph below. EC_{50} calculated as 0.43µM and 4.3µM as EC_{80}, as in Figure 20c.

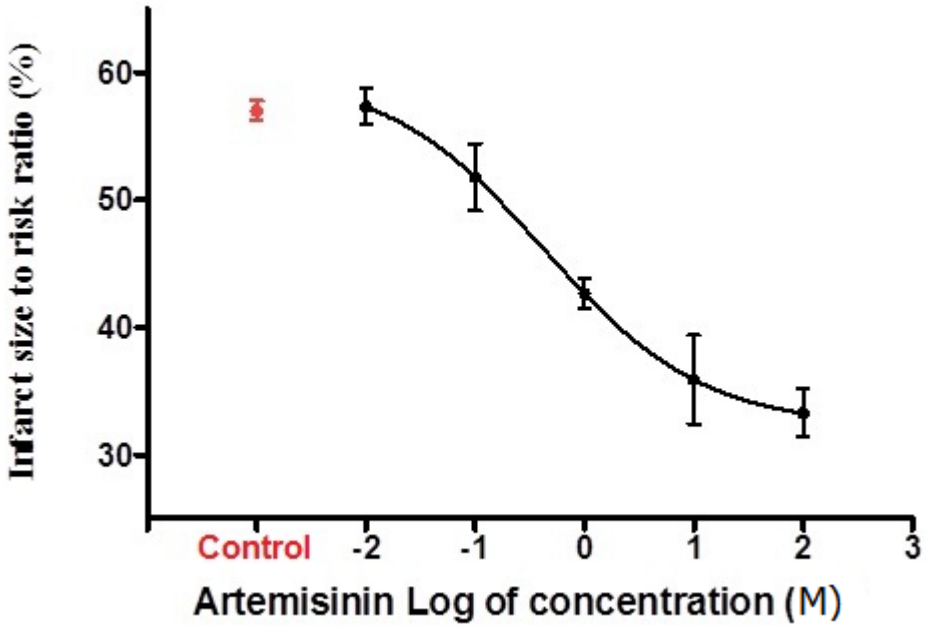


Figure 20c. *Dose response curve used to calculate the EC_{20}, EC_{50} and EC_{80} values as 0.042µM, 0.43µM and 4.3µM using values from Figure 20a. Graphs shows the effect of increasing artemisinin concentration (0-100µM) on infarct size to risk ratios in isolated perfused rat heart model of I/R. Hearts were subjected to 20 minutes stabilisation, 35 minutes ischaemia and 120 minutes reperfusion in the presence/absence of artemisinin (0-100µM)(n=4-8)*

Figure 20c shows the log dose response curve based on the infarct size to risk ratio (%) of hearts subjected to the experimental protocol of 20 minutes stabilisation, 35 minutes ischaemia and 120 minutes reperfusion.

The haemodynamic data for the individual concentrations 1µM, 10µM, and 100µM is not included. However, the haemodynamic effects for EC_{20} (0.042µM), EC_{50} (0.43µM) and EC_{80} (4.3µM) are depicted in the Figures 21-23.

3.3.3.4 The effects of artemisinin (EC_{20}: 0.042µM, EC_{50}: 0.43µM or EC_{80}: 4.3µM) treatment on LVDP in isolated perfused hearts subjected to I/R

Isolated rat hearts were stabilised for 20 minutes followed by 35 minutes ischaemia and 155 minutes reperfusion where artemisinin (EC_{20}: 0.042µM, EC_{50}: 0.43µM or EC_{80}: 4.3µM) was administered throughout reperfusion. The LVDP drastically dropped during ischaemia and increased upon reperfusion in all groups. Reperfusion generally attenuates the fall in LVDP caused by myocardial ischaemia. This occurs as a result of re-establishing flow by removing the ligation in both the control and artemisinin group during reperfusion.

There was no significant change in LVDP during stabilisation and ischaemia between all groups. Treatment with the different concentrations of artemisinin (EC_{20}:0.042µM, EC_{50}: 0.43µM or EC_{80}:4.3µM) at reperfusion generally caused no significant difference in LVDP compared to I/R. However, towards the end of reperfusion, at 120 minutes, treatment with artemisinin at EC_{20}:0.042µM concentration, resulted in an increase in LVDP compared to time matched control ($P<0.05$). This increase in LVDP was noticeable until 175 minutes (90.0±3.0% vs 70.7±2.9%, $P<0.01$, Figure 21). Treatment with artemisinin, 4.3µM and 0.43µm revealed a slight increase in LVDP compared to IR control, although this was not significant.

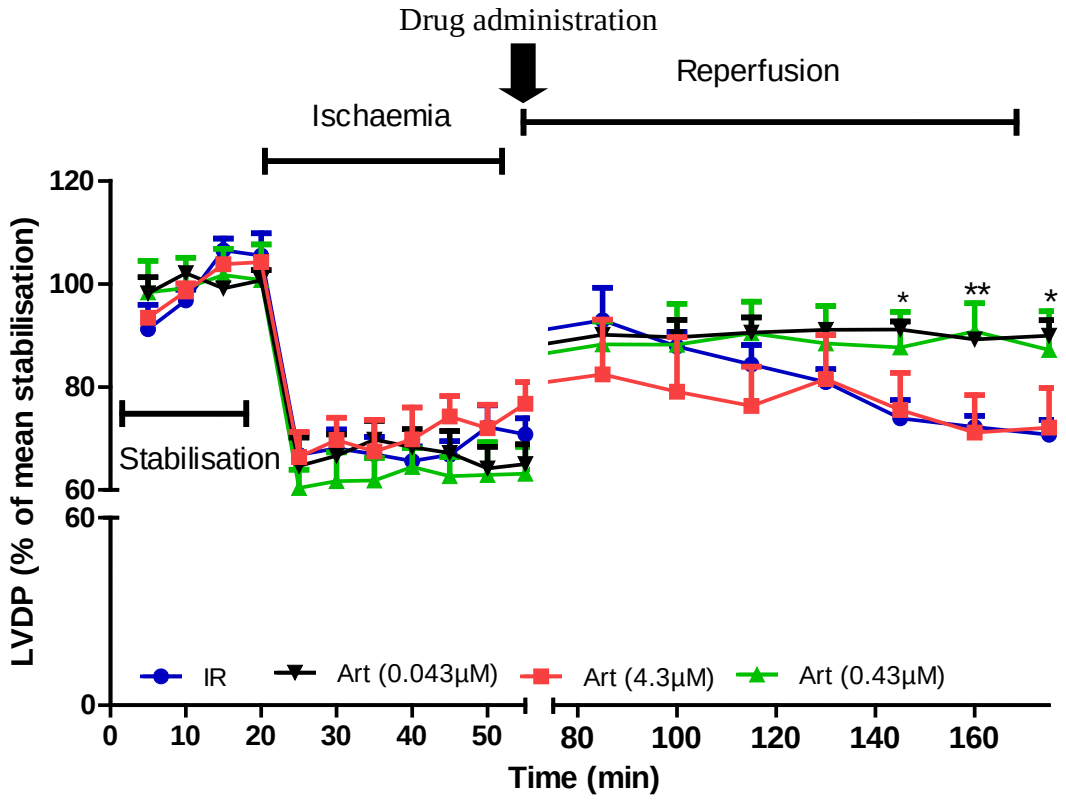


Figure 21. *The effects of artemisinin (EC_{20}: 0.042µM, EC_{50}: 0.43µM or EC_{80}: 4.3µM) on LVDP in isolated perfused hearts subjected to 20 minutes of stabilisation, 35 minutes and ischaemia and 120 minutes reperfusion in the presence/absence of artemisinin (EC_{20}: 0.042µM, EC_{50}: 0.43µM or EC_{80}:4.3µM). Data expressed as a % of mean stabilisation±SEM. artemisinin (4.3µM) *P<0.05 vs. I/R control, artemisinin (4.3µM) **P<0.01 vs. I/R control (n= 4-8).*

3.3.3.5 The effects of artemisinin (EC_{20}: 0.042µM, EC_{50}: 0.43µM or EC_{80}: 4.3µM) treatment on HR in isolated perfused hearts subjected to I/R

HR remained relatively stable with a slight increase in the drug treated group compared to untreated time matched I/R control, although did not reach statistical significance.

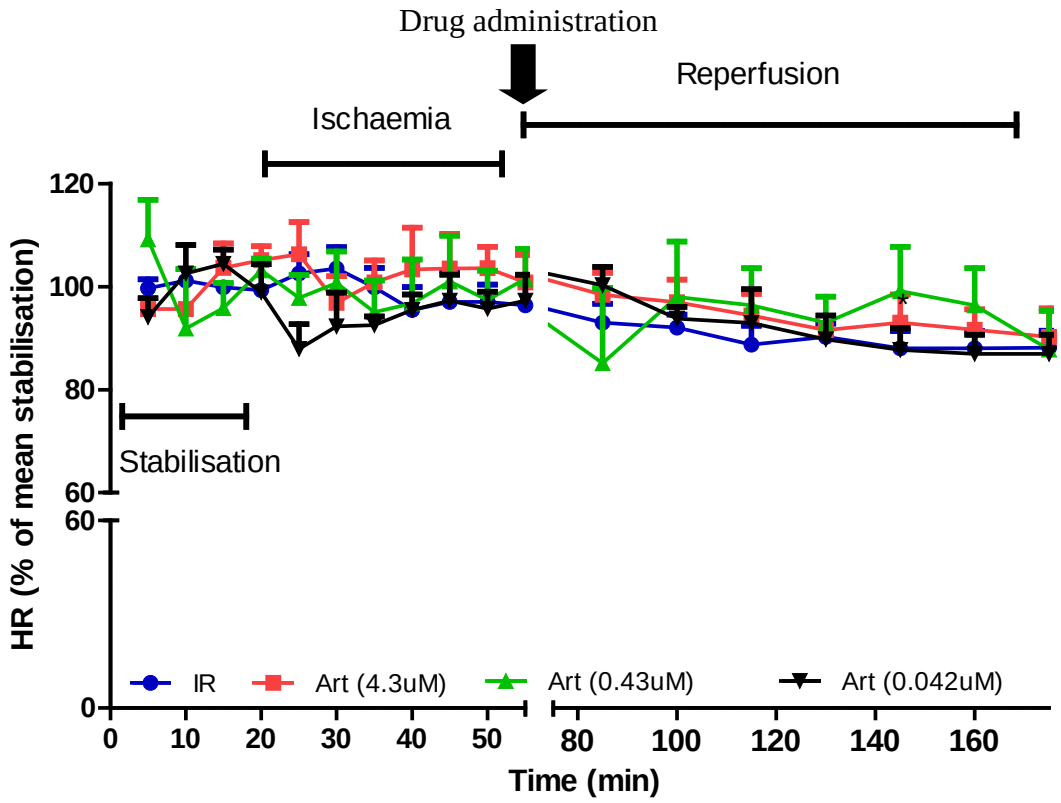


Figure 22. *The effects of artemisinin ($EC_{20:}$ 0.042µM, $EC_{50:}$ 0.43µM or EC_{80}: 4.3µM) on HR in isolated perfused hearts subjected to 20 minutes of stabilisation, 35 minutes and ischaemia and 120 minutes reperfusion in the presence/absence of artemisinin ($EC_{20:}$ 0.042µM, $EC_{50:}$ 0.43µM or EC_{80}: 4.3µM). Data expressed as a % of mean stabilisation±SEM. (n= 4-8).*

Baseline values of HR in all groups were similar in the different treatment groups during stabilisation as well as during ischaemia. During reperfusion when artemisinin ($EC_{20:}$ 0.042µM, $EC_{50:}$ 0.43µM or EC_{80}: 4.3µM) was added showed no significant difference to the untreated control groups.

The effects of artemisinin ($EC_{20:}$ 0.042µM, $EC_{50:}$ 0.43µM or EC_{80}: 4.3µM) treatment on CF in isolated perfused hearts subjected to I/R.

CF dropped during ischaemia and upon reintroducing KH buffer, CF values were shown to increase again almost to stabilisation readings. However, treating hearts with artemisinin 0.43µM and 0.042µM upon reperfusion at these two concentrations revealed changes to CF were not significant compared to untreated time matched I/R control. Artemisinin 4.3µM treated hearts showed a significant decrease in CF compared to I/R control at 160 minutes into reperfusion with artemisinin (81.7±2.8% vs. 87.1±3.2% in I/R control respectively, $P<0.05$, Figure 23).

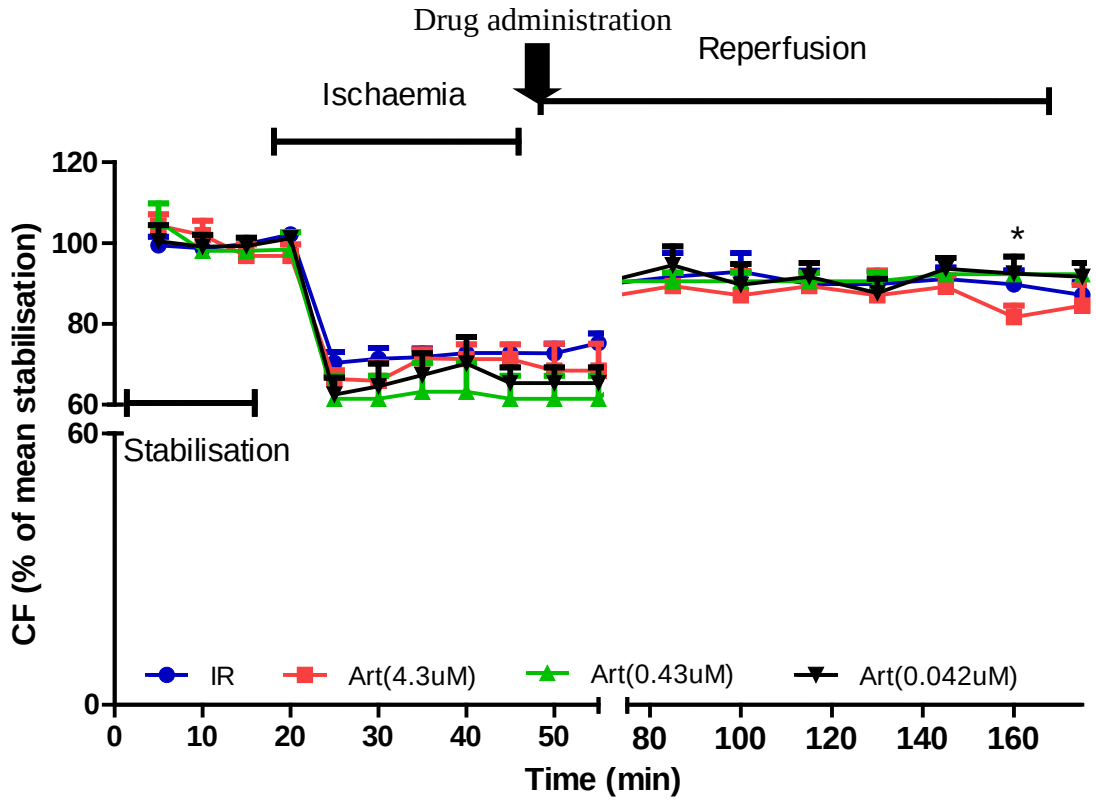


Figure 23. *The effects of artemisinin ($EC_{20:}$ 0.042µM, $EC_{50:}$ 0.43µM or EC_{80}: 4.3µM) on CF in isolated perfused hearts subjected to 20 minutes of stabilisation, 35 minutes and ischaemia and 120 minutes reperfusion in the presence/absence of artemisinin ($EC_{20:}$ 0.042µM, $EC_{50:}$ 0.43µM or EC_{80}: 4.3µM). Data expressed as a % of mean stabilisation±SEM.*P<0.05 vs I/R control) (n= 4-8).*

The infarct size in the artemisinin (4.3µM) treated group compared to I/R controls also subjected to 20 minutes stabilisation, 35 minutes of regional myocardial ischaemia followed by reperfusion for 120 minutes showed artemisinin significantly decreased infarct size (38.0 ± 2.5 % vs. 55.8±1.7% in I/R, P<0.001, Figure 24).

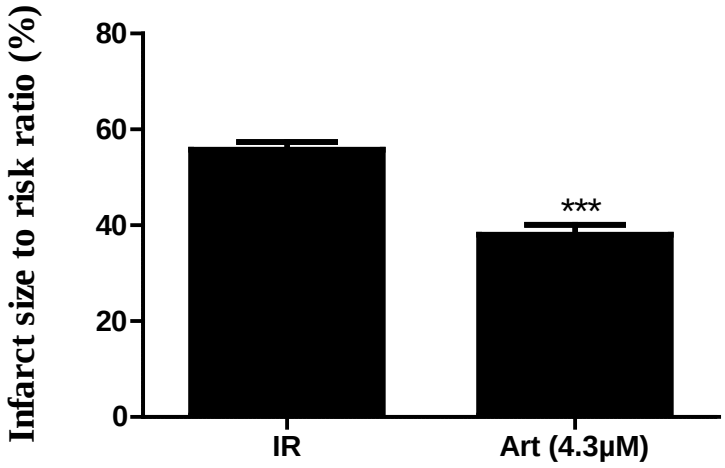


Figure 24. *The effects of artemisinin (4.3μM) on infarct to risk ratios in isolated perfused rat heart model subjected to 20 minutes stabilisation, 35 minutes ischaemia and 120 minutes reperfusion in the presence/absence of artemisinin (4.3μM) which was administered throughout reperfusion. Results were expressed as Mean±SEM (***P<0.01 vs. I/R control) (n=6).*

3.3.4 The differential expression of miRNA levels in isolated rat hearts upon I/R followed by treatment with artemisinin (4.3μM)

Studies have shown the importance of miRNA's in gene regulation with their deregulation being a common feature in a variety of diseases. More recently their expression in a variety of setting are emerging important biomarkers in cardiomyopathies (Chhabra *et al.*, 2009). Certain miRNAs that have previously been linked with myocardial injury and cancer were investigated in this study in order to evaluate their expression in I/R setting of isolated rat hearts treated in the presence/absence of the potential anti-cancer drug artemisinin (4.3μM). Hearts were subjected to same treatment protocol as the isolated rat heart experiments (20 minutes stabilisation, 35 minutes ischaemia and 120 minutes reperfusion) where artemisinin (4.3μM) was administered throughout reperfusion, following successful miRNA extraction with high purity levels. miRNA expression patterns were assessed using reverse transcription followed by qPCR and Real time PCR. Fold change in the differential expressions of miRNA-1, miRNA-27a, miRNA-133a, miRNA-133b and miRNA-155 were assessed.

As shown in the Figure 25, miRNA-1 was abundantly expressed in the untreated heart subjected to I/R setting whilst treatment of hearts with artemisinin (4.3μM) during reperfusion caused significant decrease in the expression levels of miRNA-1 compared to I/R control (0.5±0.3% vs. 51.3±11.0%, P<0.05, Figure 25). Treatment with artemisinin (4.3μM), expressed a nearly 100-fold decrease in miRNA-1 expression.

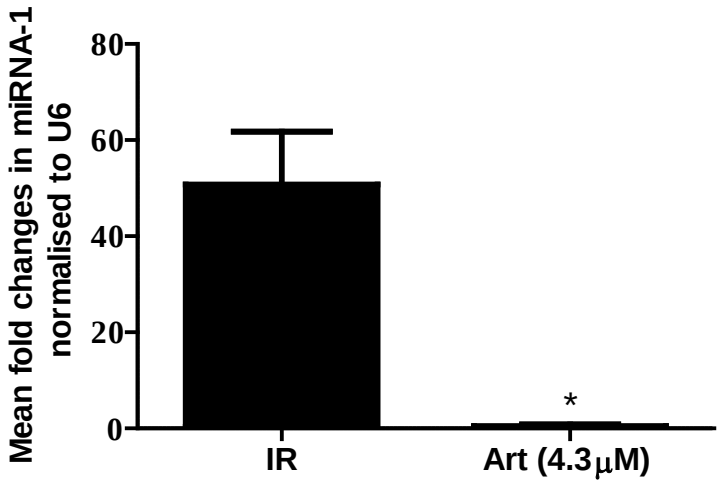


***Figure 25**. Mean fold changes in the differential expression of miRNA-1 in hearts subjected to 20 minutes stabilisation, 35 minutes ischaemia and 120 minutes reperfusion in the presence/absence of artemisinin (4.3μM) where administered throughout reperfusion. Results were expressed as Mean±SEM. (*P<0.05 vs I/R Control) (n=3-4)*

Hearts subjected to I/R showed an increase in miRNA-27a while hearts treated with artemisinin (4.3μM) showed a significant (P<0.01) decrease compared to I/R. The trend observed showed an abundant expression of miRNA-27a in the untreated heart subjected to I/R, whereas expression of miRNA-27a was very low in artemisinin (4.3μM) treatment hearts, over 20-fold decrease compared to I/R control (0.8±0.3% vs. 12.6±0.2% in I/R, P<0.01, Figure 26).

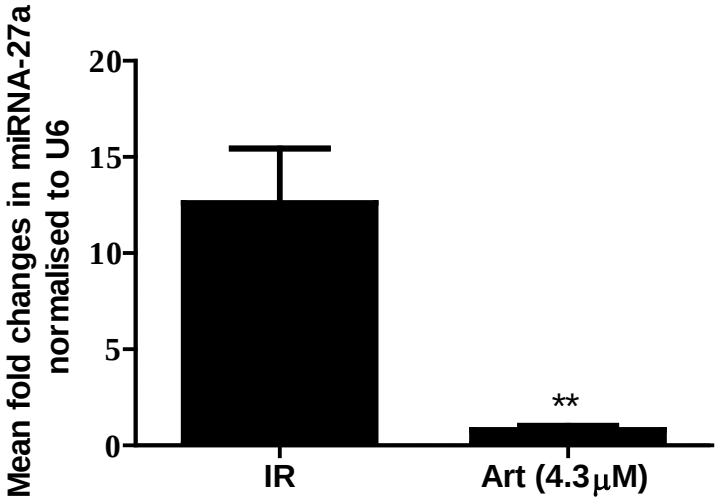


***Figure 26.** Mean fold changes in the differential expression of miRNA-27a in artemisinin (4.3μM) treated hearts compared to I/R control. Hearts were subjected to 20 minutes stabilisation, 35 minutes ischaemia and 120 minutes reperfusion in the presence/absence of artemisinin (4.3μM) administered throughout reperfusion. Results were expressed as Mean±SEM. (n=3-4)*

miRNA-133a was significantly downregulated (9-fold decrease) in artemisinin (4.3μM) treated cells compared to non-treated I/R control (19.5±3.6% vs. 174.9±13.3% in I/R control, P<0.01, Figure 27). D'Alessandra *et al*., (2010) showed upregulation of miRNA-133a to increase in studies of myocardial injury.

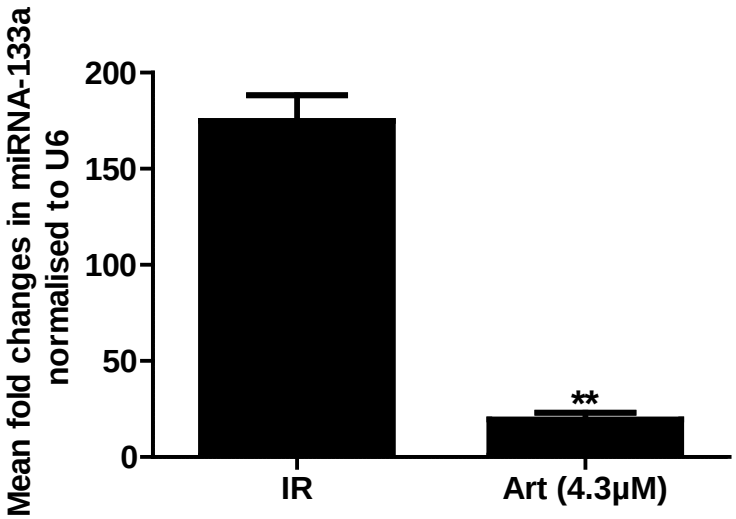


Figure 27*. Mean fold changes in the differential expression of miRNA-133a in artemisinin (4.3μM) treated hearts compared to I/R control. Hearts were subjected to 20 minutes stabilisation, 35 minutes ischaemia and 120 minutes reperfusion in the presence/absence of artemisinin (4.3μM) which was administered throughout reperfusion. Results were expressed as Mean±SEM (**P<0.01 vs. IR control) (n=3-4)*

miRNA-133b was also upregulated in the isolated perfused rat hearts subjected to I/R. Expression of miRNA-133b decreased nearly 10 fold in the artemisinin (4.3μM) treated hearts (3.8±2.2% vs. 40.0±3.8% in I/R, Figure 28) although results did not reach significance when compared to the untreated time matched control.

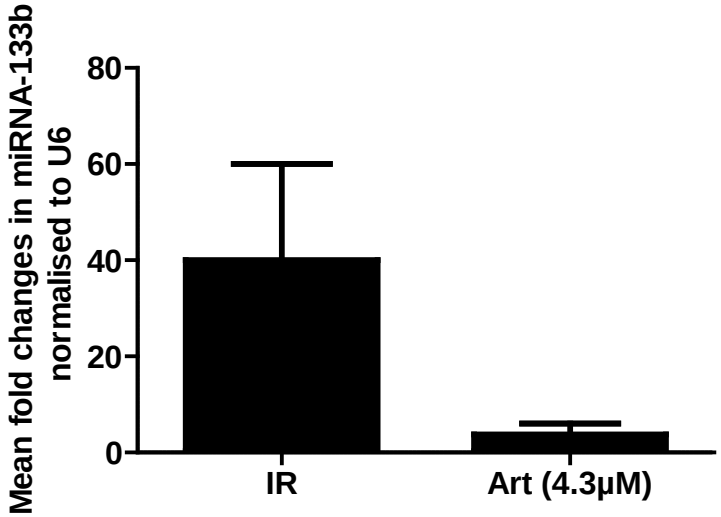


Figure 28. *Mean fold changes in the differential expression of miRNA-133b in artemisinin (4.3μM) compared to control. Hearts were subjected to 20 minutes stabilisation, 35 minutes ischaemia and 120 minutes reperfusion in the presence/absence of artemisinin (4.3μM) which was administered throughout reperfusion. Results were expressed as Mean±SEM (n=3-4)*

The expression level of miRNA-155 in the isolated perfused rat hearts subjected to I/R was downregulated by treatment with artemisinin (4.3µM) throughout reperfusion. Artemisinin (4.3µM) treated hearts showed a nearly 10 fold decrease compared to control (0.5±1.3% vs. 1.2±0.4%, P<0.05 in I/R, Figure 29)

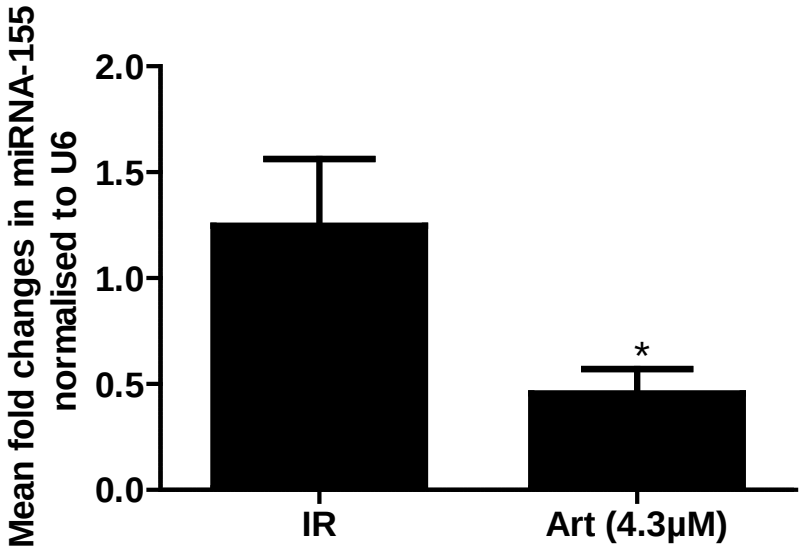


***Figure 29**. Mean fold changes in the differential expression of miRNA-155 in artemisinin (4.3µM) treated hearts compared to I/R hearts. Hearts were subjected to 20 minutes stabilisation, 35 minutes ischaemia and 120 minutes reperfusion in the presence/absence of artemisinin (4.3µM) which was administered throughout reperfusion. Results were expressed as Mean±SEM (n=3-4)*

3.3.5 Effect of artemisinin (4.3µM) on cleaved caspase-3 activity in myocytes subjected to Hypoxia/Reoxygenation (H/R)

In order to assess the cytoprotective effects of artemisinin in adult rat ventricular myocytes, changes in the levels of cleaved caspase-3 were measured via flow cytometry. Results from H/R show a significant increase in levels of cleaved caspase-3 compared to normoxic control 26.8±2.0% vs. 8.8±1.2%, P<0.001, Figure 30) Administration of artemisinin (4.3µM) throughout reoxygenation resulted in a significant decrease in the levels of cleaved caspase-3 activity when compared to the H/R control (17.1±2.0% vs. 26.8±2.0% in H/R, P<0.001, Figure 30).

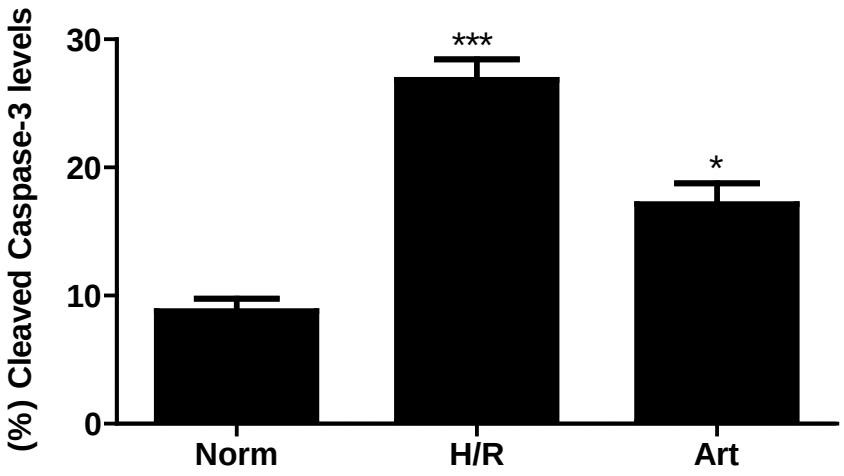


Figure 30**. % Effect of administration of artemisinin (Art) (4.3µM) on cleaved casapase-3 levels as analysed by Flow cytometry. Results were expressed as Mean±SEM (P<0.001 vs Normoxia, *P<0.05 vs H/R) (n=4-6).*

3.3.6 Changes in levels of MTT reductase activity with drug treatment following Hypoxia/Reoxygenation (H/R) in ventricular myocyte

Isolated cardiac myocytes were subjected to 120 minutes of hypoxia and 120 minutes of reoxygenation where artemisinin (4.3µM) was administered throughout reoxygenation. Following H/R, cells were subjected to MTT assay which measures the cellular effects of artemisinin by measuring the changes in the level of MTT reductase activity which determines cellular viability in the isolated adult rat ventricular myocytes.

Cells subjected to H/R showed a significant (P<0.001) decrease in cell the viability compared to normalised normoxic cells 29.3±6.1% vs. 100.0±0.0% in Normoxia respectively, Figure 31). Administration of artemisinin (4.3µM) throughout reoxygenation significantly improved cell viability compared with H/R group (66.5±6.3% vs. 29.3±6.1% respectively, P<0.01, Figure 31).

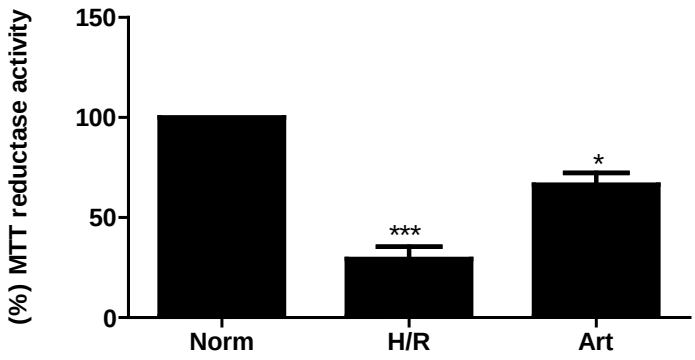


***Figure 31**. % change in MTT reductase activity compared with control in isolated rat ventricular myocytes following H/R. Cardiomyocytes were subjected to 120 minutes hypoxia followed by 120 minutes of*

*reoxygenation in the presence/absence of artemisinin (4.3µM). Results are expressed as Mean±SEM (***P<0.001 vs. Normoxic control and *P<0.01 vs. /HR) (n=6).*

3.3.7 Changes in levels of MTT reductase activity with drug treatment following Hypoxia/Reoxygenation (H/R) in HL-60 cells

The cytotoxic effects of the increasing concentration of artemisinin was investigated in order to determine a concentration at which artemisinin causes significant cytotoxicity. Artemisinin caused a dose-dependent decrease in the viability of HL-60 cells. The IC50 of artemisinin was calculated using the graphit programme as 401.9µM (0.4mM) (Figure 32a). This concentration (0.4mM) caused a significant reduction in the number of viable cells when compared to untreated time matched control in HL-60 cells (57.2%±3.2% vs. 100.0±0% in Control, P<0.001, Figure 32c).

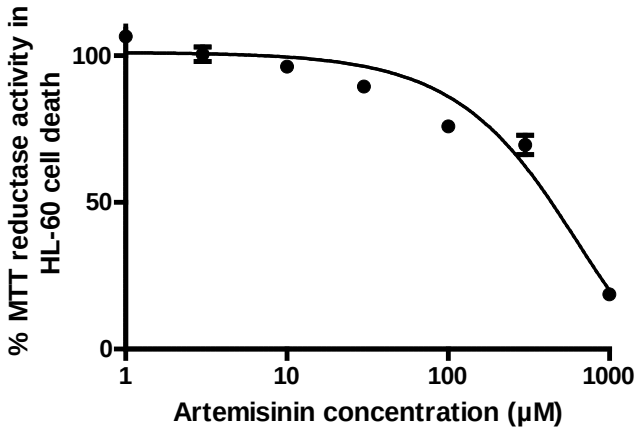


Figure 32a. *Dose response curve showing the effect of MTT cytotoxicity analysis on HL-60 cells in response to treatment with increasing concentrations of artemisinin (1-1000µM). HL-60 cells were incubated with artemisinin (1-1000µM) for 24 hours. Experiment was terminated by adding MTT solution and cells were further incubated for 2 hours until termination with lysis buffer. Plate readings were taken at 492nm and results analysed using the 4-parametric logistic analysis, Graphit software. Image of HL-60 control cells and artemisinin (0.4mM) cells, n=6 shown below (Figure 32b)*

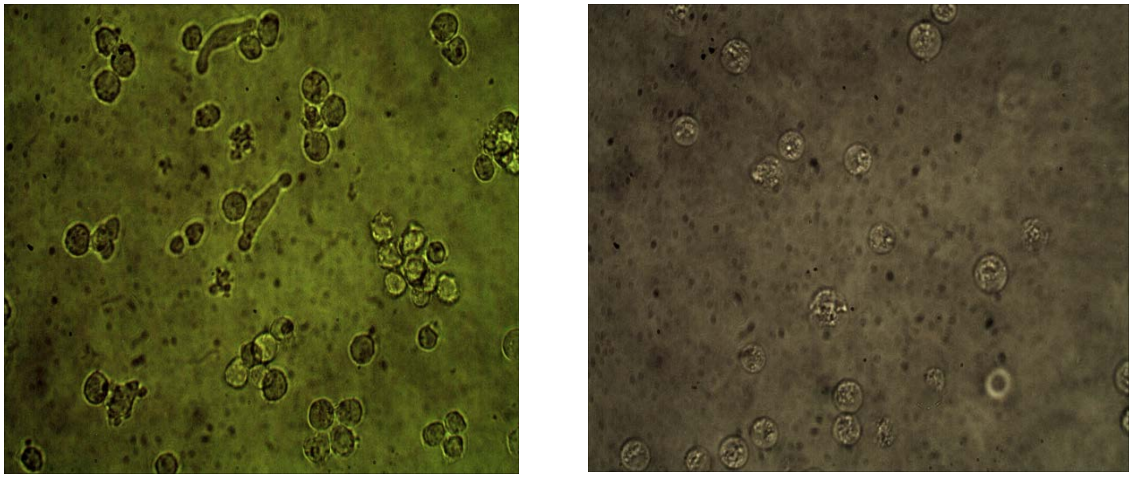


Figure 32b. *HL-60 cells control vs artemisinin treated cells. Image shows HL-60 cells to the right incubated with artemisinin (0.4mM) for picture purposes and to the left is untreated HL-60 control.*

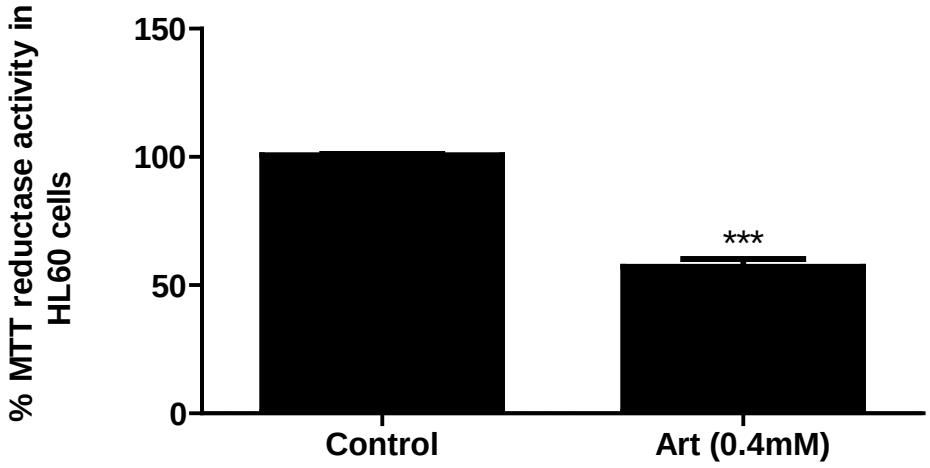


Figure 32c: *% change in MTT reductase activity compared to control in MTT cytotoxicity analysis of HL-60 cells in response to treatment with artemisinin (0.4mM). HL-60 cells were incubated with artemisinin (0.4mM) for 24 hours. Experiment was terminated by adding MTT solution and cells were further incubated for 2 hours until termination with lysis buffer. Plate readings were taken at 492nm and results analysed using the 4-parametric logistic analysis, Graphit software. Results are expressed as Mean±SEM (n=6)*

3.4 DISCUSSION

3.4.1 Reperfusion and CHD

CHDs are a number one cause of death globally, claiming 7.3 million lives in 2001 and responsible for three-quarter of all deaths worldwide (Gaziano *et al.,* 2009). Several anticancer drugs have also been associated with cardiotoxicity especially the anthracyclines and

trastuzumab (a monoclonal antibody used in combination with chemotherapy, hormone blockers, or lapatinib in the treatment of metastatic breast cancer). Pathogenesis of anthracycline-associated toxicity is well-documented, with their different mechanisms known. This has raised the need to develop pharmacological mediation that will alleviate the drug induced toxicity (Sparano *et al.,* 2002). There is an imperative need to develop a drug/adjunctive therapy to anti-cancer treatment that do not exarcerbate cardiac injury particularly in cancer patients who may have underlying ischaemic heart disease. Ischaemic heart disease is one of the prevalent manifestations of CHD often resulting as a consequence of oxygen and nutrient deprivation to parts of the heart which leads to severe tissue damage which may also lead to death (Palcher *et al.,* 2007; Hadjipanayi and Schilling 2013). Several clinical interventions are used to currently limit or prevent the extent of a cardiac event from occurring (Niccoli *et al*., 2014). Ultimately, however patients with CHD will require reperfusion treatments. Reperfusing the tissue is an effective approach to managing ischaemia and in the long run curbs mortality (Xu *et al.,* 2014). In spite of its clinical benefits, concerns have been raised as to the effectiveness of reperfusion treatments due to injury caused as a result of this mediation to cells/tissues (Yellon *et al.*, 2000).

With research in the past showing that by restoring blood flow to the deprived ischaemic area by either therapeutic means or surgical procedures (an effective mechanism of salvaging reversible cardiac injury), reperfusion poses a potential risk of early patient mortality in the patients receiving the treatment (Collard *et al.* 2001; Lee *et al.* 1995). While the blood flow relieves ischaemia, it may also result in cardiac damage, oxidative stress and complications called reperfusion injury (Gibson *et al.* 2009). Hence, the effect was termed as the double edged sword (Braunwald and Kloner 1985). Reperfusion injury maybe accompanied by several irreversible pathologies such as microvascular injury (stunning) and cellular damage known as reperfusion injury (Braunwald and Kloner 1985). Limiting the impact of I/R injury is of vital importance given the death figures relating to cardiovascular related mortality in the world.

3.4.2 Artemisininin alleviates myocardial injury

There is evidence in a myocardial model of I/R injury using Wistar rats that artemisinin possesses some cardioprotective effects that can salvage cardiac myocytes by limiting infarct size development (Sun *et al*., 2007). Artemisinin, also known as *Qinghaosu,* is an active component of the Chinese medicinal herb *Artemisia annua* (sweet wormwood*)* (Efferth *et al*., 2008) which is a popular treatment for flu and fever in ancient china (Crespo-Ortiz and Wei

2012). Artemisinin is presently administered as combination therapy against malarial infections worldwide (Dondorp *et al.*, 2010 and Pasvol 2005). Studies have also shown artemisinin to be effective against pathophysiological conditions such as parasitic infections, viral infections, autoimmune diseases and a variety of cancer types (Harter and Michel, 2012; Mirshafiey *et al.*, 2006; Ho *et al.*, 2012; Hou *et al.*, 2008; Xu *et al.*, 2007).

In our results we were able to show that artemisinin confers protection in a dose dependent manner by alleviating the induced myocardial I/R injury. Artemisinin was shown to be cardioprotective in this model of I/R at concentrations as low as 0.1µM where it significantly attenuates the development of myocardial infarction in isolated perfused rat hearts.

Our results are consistent with the findings of Sun *et al.*, (2007), which showed artemisinin at 10µM and 100µM alleviated myocardial infarction in Wistar rats which, they speculated could be associated with its functions of anti-oxidation and scavenging of free radicals although this was not shown in their paper. No intracellular signalling pathways were actually investigated in Sun's paper. Their data showed 10µM and 100µM significantly improved myocardial function and increased blood flow after ischaemia which was concurred in our study where we observed an increase in CF at 105 minutes into reperfusion with artemisinin (4.3µM) when compared to the untreated control. Interestingly, Sun *et al*.,(2007) showed an increase in LVDP compared to non-treated I/R control whereas LVDP in our artemisinin (4.3µM) treated hearts showed no significant difference. However, administering artemisinin at lower concentrations of 0.43µM showed slight increase in LVDP towards the end of drug treatment however this increase was not significant in comparison to the control. A significant increase in LVDP was observed with 0.042µM artemisinin administration compared to non-treated IR control towards the end of reperfusion. This can be explained as a result of the anticholinergic effects of artemisinin which may evoke anticholinergic mediated tachycardia in isolated guinea pig atrial cells (Hara *et al*., 2007). Dose dependent acute effects of artemisinin in heart subjected to I/R remain relatively unaffected in our studies. Changes in HR and CF as well as artemisinin treatment ($EC_{20:}$ 0.042µM, $EC_{50:}$ 0.43µM or EC_{80}: 4.3µM E) resulted in no significant differences between the treatment groups. Artemisinin appears to bring about its effect without significantly altering haemodynamics as observed with results from 4.3µM and 0.43µM concentrations of artemisinin. It has been suggested clinically that, enhancement of vagal tone with administering anticholinergic drugs can lead to atrial fibrillations with the documented effect being variable or even spontaneous (Chen and Tan 2007). Although artemisinin is shown

to have minimal side effects, functional studies have reported changes in action potential and inotropic responses thus suggesting it may have possible cardiac influences (Hara *et al.,* 2007). Which was represented in our normoxic hearts whereby perfusing the hearts with artemisinin 4.3µM, showed an increase in HR with a time lag of 10 minutes upon drug administration for the drug to reach the heart and show a response. The drug administration revealed a gradual increase which became more pronounced towards the end of the treatment. CF was also significant increased at some time points i.e. upon perfusion and towards the end of artemisinin treatment. This effect although not previously reported may be explained in terms of the diuretic effect the artemisinin's, sodium artesunate, an artemisinin derivative has been shown to have diuretic effects as well as natriuretic effects in patients with malaria (Zaki *et al.,* 2011). Arteether, another artemisinin derivative shown to have no cardiovascular effects in rats has been shown to have minor diuretic effects (Kar *et al*., 1989). Our findings agree with this study by showing no significant effect on LVDP throughout artemisinin (4.3µM) administration. Diuretic therapy however has important antianginal effects and as a result of this effect on intravascular volume it is used in the treatment of high blood pressure (Parker *et al.,* 1996). Short acting diuretic effects are however shown to have effects on HR variability in patients with heart failure (Parker *et al.,* 1996; Tomiyama *et al.,* 1999) which disagrees with our findings as this was observed in the normoxic hearts. Diuretics have been associated with increase in HR resulting in tachycardic episodes (Tomiyama *et al.,* 1999). This suggests a possible increase in HR may be observed due to the HR variability caused by the diuretic effect of artemisinin. However in dogs, artemisinin derivatives, artesunate and artemeether administered over a 3 month period while demonstrating a spectrum of toxic changes was followed by a decrease HR in the experimental dogs (Yin *et al.,* 2014). Efferth and Kaina (2010) however have explained considerable toxicities may be found in animal studies but not in human studies. This is however as a result of drug differences such as mode of administration can lead to better drug safety and profiles. By adminstering artemeether or arteether intramuscularly, Efferth and Kaina (2001) observed no effect on the variety of haemodynamic parameters. Artemisinin and its derivatives are reported to be well tolerated and relatively safe in different models (Hara *et al.,* 2007).

Since time immemorial, herbs have been shown to be used for medicinal purposes. Claims have been on the earliest reference of earliest reference of use of medicinal herbs as a cure for a disease was found in Ebers Papyrus (2600 BC), other references date back to herbs in Rig Veda (period estimate between 3500-1800 BC). Medicinal plants/herbs are widely used and

have been shown to possess antiplatelet, hypolipidemic, anti-inflammatory, hypoglycemic and hypotensive actions in a variety of setting such as congestive heart failure, systolic hypertension, angina pectoris, atherosclerosis, cerebral insufficiency and arrhythmia.

Chinese herbal plant *Carthamus tinctorius L*. (safflower), is widely used in preventing and treating cardiac disease in clinical settings where it is shown to alleviate ischaemic injury *in vivo* and *in vitro.* Other medicinal plants include *Sini Decoction* (SND) administered against myocardial I/R injury where it is shown to ameliorate mitochondrial oxidation injury in Kun Ming mice where cardioprotection was presented as limiting mitochondrial swelling, lactic acid content of myocardium and increase in the expression of Mn SOD miRNA with SND treatment (Zhoa *et al.,* 2008). A study by Cheng *et al*. (2005) showed the inhibition of lipid peroxidation, augmentation of endogenous antioxidants and improving myocardial metabolism upon administering *Curcumin* against myocardial injury in rat models of myocardial I/R injury. Curcumin presented decrease in infarct size and the aforementioned mediations.

3.4.3 Differential expression of miRNAs in response to I/R and treatment with artemisinin.

Vast amount of studies have shown apoptosis to be mediated via several pathways including the intrinsic and extrinsic pathways (Li *et al*., 2013) As previously mentioned, there is therefore a need for reliable, specific and sensitive biomarkers that can detect sub-clinical acute cardiac drug induced injury (Li *et al*., 2013; Jiang *et al.*, 2014). Studies have identified unique miRNA signatures in disease conditions such as chronic cardiovascular diseases where such as atherosclerosis serum levels of miRNA were quantitatively measured using PCR in peripheral bood samples of pre-atheroscelerotic patients and marked changes were reported. Jiang *et al.* (2015) suggested by integrating specific patterns of miRNA with BNP or cardiac troponin, diagnosis of cardiovascular disease will significantly improve.

Caspases and mitochondrial involvement are also well researched in terms of cellular signalling, survival and death. However, until recently very little was known about miRNA-mediated apoptotic pathways in myocardial injury. Several studies have showed miRNAs to be important regulators of apoptosis and in cardiac injury. Depending on the nature of the targeted genes, miRNAs can either be pro-apoptotic or anti-apoptotic, although over 93% of known miRNAs in vertebrae have been shown to have at least one target gene related to cell death and survival (Yang *et al.,* 2009).

Promising evidence has also shown the importance of the role of miRNAs in cardiovascular disease (Ha 2011; Xiao and Chen 2010). Elevated levels of biomarkers in the blood do not diagnose underlying mechanisms of diseases but are however good indicators of myocardial damage (Salic and Windt 2012). Recently, miRNA's such as miRNA-21, miRNA-92 and miRNA-101 are being characterised and used as biomarkers in several cardiomyopathies which are deregulated in dilated cardiomyopathy (Ikeda *et al*., 2007). Deregulation of miRNA-208 expression is also associated with a poor clinical outcome and therefore represents a potential prognostic marker of human dilated cardiomyopathy (Satoh *et al*., 2010). It is well established that the expression of many miRNAs changed significantly in diseased myocardium with studies showing altered expression of miRNA-1 and miRNA-133 to be associated with human heart failure (Care *et al.,* 2007; Yang *et al.,* 2007).

In our study we investigated the role of miRNA-1 in modulating artemisinin induced cell survival and differential expression in response to I/R injury. miRNA-1 can act as both a pro and/or anti-apoptotic factor in cardiac I/R injury (Gidlof *et al.,* 2011; and Tang *et al.,* 2009). miRNA-1 is closely related with I/R injury in a rat model (Tang *et al*., 2009) and has been well documented to be a biomarker for predicting acute myocardial infarction in humans too where an upregulation in miRNA-1 is seen in patients with myocardial infarction (Ai *et al*., 2010). Over-expression of miRNA-1 has been shown to increase in response to oxidative stress. In our I/R control group however, levels of miRNA-1 were evidently upregulated compared to the cardioprotective drug treatment (artemisinin), previous studies have shown cardioprotective drugs to express a decreased level of miRNA-1 compared to control which was significantly higher in our result as well as previous studies(Tang *et al*., 2009; Wu *et al*., 2011). Cheng *et al.,* (2010) showed a 200 fold increase in the level of miRNA-1 in Sprague Dawley rats with the introduction of Triton-100, a compound primarily administered to induce cellular necrosis (Cheng *et al.,* 2010). This suggests administering cardiotoxic drugs may lead to upregulation of miRNA-1 which agrees with previous finidngs. Treatment with artemisinin in our results showed a significant 4-fold decrease in the expression of miRNA-1. Research by Xu *et al*., (2007) showed decreased levels of miRNA-1 favours cardiomyocyte survival which corresponds to the effect seen in the different models upon administering artemisinin. miRNA-1 has been well implicated in the induction of apoptotic or necrotic cell death in different organs and in circulating blood plasma (Wu *et al*., 2011). We can therefore postulate the decrease in the expression of miRNA-1 levels observed in our study may be associated with the cardioprotection offered by artemisinin.

miRNA-27a has been shown to play a pivotal role in the pathogenesis of cardiac hypertrophy and dysfunction as implicated in previous studies (Divakaran and Mann 2008; Nishi *et al.*, 2010; Da Costa Martins and De Windt 2012). miRNA-27a has also previously been implicated in a variety of cancer types such as breast cancer, pancreatic cancer, prostrate cancer, leukaemia and so on (Mertens-Talcott *et al.*, 2007; Liu *et al*., 2009; Fletcher *et al.*, 2012; Zhoa *et al.*, 2011; Ma *et al.*, 2010). Upregulation of miRNA-27a has been shown to induce caspase-dependent and independent apoptosis in human embryonic kidney cells (Chhabra *et al*., 2009). Upregulation in the levels of miRNA-27b in Smad4 (gastrointestinal malignancy-specific tumor suppressor gene) knockout mice has been shown to also induce cardiac hypertrophy and heart failure while its supersession led to the inhibition of the hypertrophic cell growth (Wang *et al.*, 2011). miRNA-27a is often implicated in breast cancer as an oncomiRNA (Mertens-Talcott *et al.*, 2007). It has previously been implicated in human gastric adenocarcinoma where its downregulation was shown to confer sensitivity of drugs on gastric cancer cells (Liu *et al*., 2009). miRNA-27a levels were partially downregulated by administering artemisinin although data did not reach statistical significance. Down regulation of miRNA-27a also suppresses the growth, colony formation and migration of pancreatic cells, prostate cancer and various other cancer types (Fletcher *et al.*, 2012; Zhoa *et al.*, 2011; Ma *et al.*, 2010).

It is evident that in cardiomyocytes apoptosis is a key event in I/R studies and interventions are constantly being developed to salvage the situation. By administering artemisinin in this study, we have been able to limit myocardial injury which suggests artemisinin has a great potential as a therapeutic agent against myocardial I/R injury. We also investigated the differential expression of certain miRNAs in the cardiomyocytes subjected to I/R injury and upon treatment with artemisinin. Some miRNAs have been implicated in cardiovascular disease such as miRNA-1 shown to reduce myocardial infarction through repressing certain apoptotic genes and up-regulating anti-apoptotic genes. Pro-apoptotic miRNAs such as miRNA-133a are important potential targets too (He *et al*., 2011). In another study, circulating miRNA-1, miRNA-133a, miRNA-499, and miRNA-208a were found to increase in patients with acute MI (Sodha *et al.*, 2009). This is similar to the expression patterns which we detected in miRNA-1 and miRNA-133a in rats subjected to acute myocardial injury.

miRNA expression varies among tissues; miRNA-1, miRNA-133a, and miRNA-133b are strongly expressed in the heart and skeletal muscle and are regarded as cardiac-specific miRNA's (Lopez-Neblina *et al.*, 2005; Chen *et al.*, 2006). Both miRNA-1 and miRNA-133

have been shown to play a crucial role in regulating cardiac hypertrophy both *in vivo* and *in vitro* are highly expressed in the heart muscle (Dong and Yang 2011).

miRNA-133b on the other hand is a versatile pro-apoptotic molecule (Ratovitski 2013). It is currently being explored as a therapeutic target for anti-cancer treatment with increased expression of THP1 macrophages (Human monocytic cell lines) and HL-60 (HeLa cells) following innate immune activation by members of the Toll-like receptor (TLR) family (Arcila 2010). With the potential of artemisinin as a possible anti-cancer therapy we anticipated a decrease in. Expression levels of miRNA-133b in our artemisinin treated heats did show a decrease in miRNA-133b expression however results did not reach significance when compared to the controls.

miRNA-155 has been shown to be consistently up-regulated during acute myocarditis in both humans and mice models (Corsten *et al.,* 2012). miRNA-155 has been associated with heart failure, inducer of pathological cardiomyocyte hypertrophy, down modulates inflammatory cytokine production and mediate inflammatory response (Seok *et al.,* 2014) which agrees with our I/R hearts. Artemisinin treated hearts interestingly, reversed the miRNA-155 expression by 10 fold compared to I/R control. Similarly, Seok *et al.,* (2014) in their experimental mouse model of cardiac hypertrophy showed loss of miRNA-155 prevents progression of heart failure and extends the survival in mice. Our results suggest artemisinin down-regulates miRNA-155 which has been shown to suppress cardiac hypertrophy in response to stressors and by therapeutically inhibiting endogenous miRNA-155. Previous studies have established artemisinin has the ability to suppress cardiac hypertrophy in primary cultured rat cardiac myocyte by activating genes involved in the pathogenesis of cardiac remodeling and heart failure (Xiong *et al*., 2010).

Nazari-Jahantigh *et al.,* 2012 showed miRNA-155 targets *Bcl6* (ordinarily an unstimulated macrophage) which promotes pro-inflammatory activation. miR-155 has been shown to mediate the suppression of *Bcl6* (ordinarily an unstimulated macrophage)which causes the progression of atherosclerosis by influencing macrophages to atherogenic activation which is vital for associated inflammatory response (Nazari-Jahantigh *et al.,* 2012). Therefore miRNA-155 up-regulates inflammatory macrophages amplifying vascular inflammation and other coronary heart diseases. These findings agree with the outcomes from our results.

3.4.4 Artemisinin 4.3μM improves cell viability of cardiomyocytes subjected to hypoxia/reoxygenation (H/R) injury.

In this study, the isolated cardiomyocytes were subjected to 2 hours of hypoxia and 2 hours of reoxygenation where artemisinin (4.3μM) was administered throughout the reoxygenation period. Following H/R, the cells were subjected to MTT assay which gives an indication of cell viability (Stockert *et al.,* 2012).

Administering artemisinin (4.3μM) at reperfusion showed improvement in myocyte viability following H/R, this is indicative of the potential of artemisinin to salvage H/R induced injury in adult ventricular myocytes. Additionally, artemisinin has the ability to attenuate and prevent cell damage in response to ischaemic and hypoxic injuries according to our study. Xiong *et al.*, (2010) similarly showed artemisinin blocked angiotensin II-induced cardiac hypertrophy *in vitro* in a concentration-dependent manner thus inhibiting cell damage. The effect of artemisinin on cardiac hypertrophy was blocked after IκB-α which was silenced by transfection of cardiomyocytes with IκB-α siRNA. The study suggests artemisinin could be an effective preventive and therapeutic agent against cardiac hypertrophy and heart failure by inhibiting NF-κB signal pathways (Xiong *et al*., 2010).

3.4.5 Artemisinin attenuates myocardial injury in cardiomyocytes subjected to H/R injury by reducing the level of cleaved caspase-3 activity.

It is well established that ischaemia results in necrotic tissue and/or apoptosis (Iliodromitis *et al.*, 2007; Morin *et al*., 2009). Cleaved caspase-3 is a pivitol effector caspase in apoptotic signalling (Sakamaki and Satou 2009; Lu and Chen 2011). Studies have shown that activation of caspase-3 maybe as a result of mitochondrial oxidative stress which causes it rupture and release cytochrome c into the cytosol forming complexes known as apoptosomes (Montaigne *et al.*, 2012; Cui *et al.*, 2012 and Dai *et al*., 2014).

Our study showed treatment with artemisinin (4.3μM) protects the heart by salvaging myocytes committed to apoptotic pathways. Artemisinin (4.3μM) significantly improved cell viability in isolated adult rat cardiomyocytes compared to the non-treated H/R group control. Hypoxia has been shown to stimulate cellular shrinkage which has been previously identified as a feature of ischaemic caspase-dependent cell death (Shinzawa and Tsujimoto 2003). This explains the effects seen in the H/R group and may suggest artemisinin is a potential scavenger that reverses this effect.

Cardiomyocyte death occurs during ischaemia however more myocytes maybe damaged as a result of reperfusion injury as has been shown in previous studies (Baines *et al*., 2011; Braunwald and Kloner 1985). This explains the effects seen in the I/R hearts and H/R groups. These studies validate that the hearts have successfully undergone ischaemia and reperfusion. Administration of artemisinin causes a significant decrease in cleaved caspase-3 activity. With apoptosis being a reliable marker of cellular injury, evaluating therapeutic agents such as artemisinin using cleaved caspase-3 is imperative especially with artemisinin's potential. Treatment with artemisinin during reperfusion has shown much promise in terms of cardioprotection against I/R in isolated rat hearts therefore investigating the effect of cleaved caspase-3 in isolated cardiomyocytes re-affirmed artemisinin's anti-apoptotic potential in isolated cardiomyocytes subjected to H/R injury.

3.4.6. Artemisinin is potent against HL-60 cancer cells

Many reports on artemisinin show potent cytotoxic effect in a variety of cancer cells (Crespo-Ortiz and Wei 2012, Qaderi *et al.,* 2013. Due to artemisinin and its derivatives structurally possessing an endoperoxide bridge, it is thought to lead to the production of free-radical species which in turn explains its cytotoxicity in cancer cells (Beekman *et al.,* 1996).

As an additional study, we investigated the anti-cancer activity of artemisinin in HL-60 cells. Artemisinin was established as a potent anti-cancer drug at 0.4mM, the IC_{50} which shows strong evidence that artemisinin induces apoptosis in HL-60 cells. Our results are in agreement with the study by Jones *et al*., (2009) who showed artemisinin–acridine hybrids display promising antitumour activity in HL-60 by inducing cell death by apoptosis. Artemisinin linked to acridine was shown to enhance antitumour activity in the HL-60 leukaemia cell line (Lipinski *et al.,* 2000). The apoptotic effect of artemisinin in cancer cells has been associated with internalisation of iron which explains artemisinin's selective toxicity towards cancer cells (Lipinski *et al*., 2000). Cytotoxicity is increased in cancer cells with high cellular iron content as this makes the tumour cells more sensitive to oxidative stress (Lipinski *et al*., 2000 and Efferth *et al.,* 2003). This effect is absent in normal non-tumouric cells. Efferth *et al*., (2004) reported very minimal cytotoxicity as a result of artemisinin treatment in normal non cancer cells. However Efferth *et al*., (2004) also suggested that other iron-related genes may also be involved in the cellular response and carcinogenesis of the artemisinin's such as mitochondrial aconitase and ceruloplasmin (ferroxidase) which were are identified as important targets of the cellular response to artemisinin's's when administered with ferrous iron (Efferth *et al*., 2004).

Observed cytotoxicity was potentially as a result of artemisinin treatment as well as oxidative stress which accounts for the high rise in ROS production (Barrera 2012).

Willoughby (2002) also showed Artemisinin at 0.3mM significantly down-regulated the expression of CDK2 and CDK4 (both important modulatory proteins in cell division) on LNCaP prostate cancer. In cancer cells, CDKs are overactive, causing unregulated proliferation in a variety of cancer types (Yoon *et al.*, 2012). This suggests artemisinin has the capacity to change the function of certain transcription regulators leading to the selective loss of CDK4 gene expression (Willoughby 2002). In Human colon cancer (HT29) the artemisinin's have been found to activate the BAX family to induce the release of cytochrome c, leading to apoptosis in cancer cells (Riganti *et al.*, 2009).

In our study we have shown artemisinin's important function as an anti-apoptotic/pro-survival agent in cardiac cells subjected to I/R injury and also as a pro-apoptotic agent against HL-60 cancer cells. In this study we observed artemisinin as an effective chemopreventative agent against HL-60 cancer cells, with other studies claiming its cytotoxicity against several other cell lines (Hou *et al*., 2008). We also observed artemisinin's anti-apoptotic function in cardiac cells where it expresses cardioprotective properties in both healthy and I/R hearts. In HL-60 cancer cells artemisinin was shown to decrease cellular viability whereas in isolated cardiomyocytes despite simulated myocardial injury as a result of induced I/R or H/R artemisinin was shown to improve viability and salvage injured myocytes by preventing a multitude of processes from occurring which may trigger cell death. Studies have shown anti apoptosis and cellular survival serve to counteract programmed cell death in cells and the two processes are never mechanistically distinct (Portt *et al*., 2010). However, artemisinin in our study was revealed to have a dual but conflicting function in cardiomyocytes and in HL-60 cells, artemisinin is suggested to activate apoptotic signalling complexes that initiate death in HL-60 cancer cells as well as capable of salvaging the injured myocardium. This signifies the importance of artemisinin and its potential particularly in cancer patients with underlying ischaemic heart disease.

3.5 CONCLUSION

With novel research on artemisinin, we can conclude artemisinin offers a promising remedy in combating a variety of human diseases beyond malaria. Our results showed artemisinin has the potential to salvage cells marked for apoptosis and reverse the effect of myocardial injury also.

Results show artemisinin decrases infarction, increases viability, decreases caspase-3 activity in isolated perfused hearts and cardiomyocytes subjected to I/R injury. Hearts treated with artemisinin upon reperfusion/reoxygenation revealed a decrease in the expression of miRNAs associated with apoptosis/myocardial injury. Results from HL-60 cells also confirmed artemisinin's selective cytotoxicity against HL-60 cancer cells. Artemisinin was observed to be pro-apoptotic in HL-60 cancer cells and anti-apoptotic in the myocardium thus suggesting artemisinin's vital importance particularly in cancer patients with underlying comorbidities such as ischaemic heart disease where artemisinin has the potential to be used as an anti-cancer agent without causing drug induced cardiotoxicity. Further studies were conducted to investigate cell signalling pathways associated with the observed cardioprotection which will be discussed in subsequent chapter.

Chapter 4

4 ARTEMISININ ATTENUATES MYOCARDIAL ISCHAEMIA REPERFUSION INJURY VIA RECRUITMENT OF THE PI3K-AKT-P70S6K/BAD CELL SURVIVAL PATHWAY.

4.1 INTRODUCTION

Ischaemic heart disease (IHD) is a persistent public health burden due to its wide prevalence and high impact on morbidity and mortality worldwide (Chilton 2004; Hausenloy and Yellon 2013; Silachev *et al*., 2014). IHD is characterised by a restriction in blood flow to the coronary arteries which is associated with oxidative stress, where the mitochondria act as a major source of ROS often resulting in acute or severe tissue injury, depending on the duration and magnitude of the ischaemic insult (Eltzshig and Eckle 2011; Kalogeris *et al*., 2012). Restoration of blood flow to an ischaemic organ which we have previously discussed in detail is in order to maintain tissue viability is accompanied with 'reperfusion injury' to the ischaemic organ (Yellon and Haunsenloy 2013; Silachev *et al.*, 2014).

Previous studies have shown artemisinin suppresses myocardial hypertrophy in response to aortic banding in Sprague Dawley rats and that artemisinin it decreases myocardial injury in the rat heart model of ischaemia reperfusion (I/R) injury as discussed in detail in previous chapter however the intracellular processes mediating this protection in the heart was not fully investigated (Xiong *et al.,* 2010; Sun *et al*., 2007). Gu et al. (2012) have shown artemisinin is cardioprotective and inhibits ventricular remodelling via the inhibition of the NF-κB Pathway which results in inhibition of inflammation and prevents cardiac dysfunction. They further speculated artemisinin may inhibit MMP-2 and MMP-9 (inflammatory cytokines) expression levels although the mechanism remains unclear. (11). However, studies have suggested that the activation of the NFkB pathway is usually a causal effect in cardiac hypertrophy response (Gu *et al*., 2012).

Artemisinin which has been shown to be effective in a variety of pathophysiological conditions such as malarial infections (Krishna *et al.,* 2006), viral infections (Harter and Michel, 2012), inflammation (Mirshafiey *et al.,* 2006) and cancer chemotherapy, *in vitro* and *in vivo* (Ho *et al*., 2012; Hou *et al.,* 2008; Luo *et al*.,2014) is a drug with multiple potential.

However in anti-malarial and anti-cancer studies where interest in artemisinin lies, it has been shown to exacerbate the formation of free radicals by cleaving the endoperoxide bond within its structure (Ho *et al.,* 2014; O'Neill *et al.,* 2010). This has led to several investigations associating artemisinin with different signalling pathways such as MAPK, NF-κB, Wnt, β-catenin, PI3K pathway in a range of different models and cell lines (Mirshafiey *et al*., 2006; Xu *et al*., 2007; Ho *et al.*, 2012;Cheng *et al*., 2013; Li *et al.*, 2013) .

Studies have demonstrated that the recruitment of the PI3K-Akt cell survival pathway, also referred to as the RISK pathway, as an important target for cardioprotection which is mediated via recruitment of pro-survival kinases such as Akt, p70s6k, and BAD (Li *et al.*, 2011; Hausenloy *et al.*, 2005). Phosphorylation of down-streaming targets of Akt such as p70s6k is responsible for cell growth and proliferation (Courtney *et al.*, 2010; Ye *et al.*, 2010). Danial (2008) reported phosphorylation of BAD $_{(Ser136)}$, an anti-apoptotic protein and a downstream target of Akt, as an important mediator of cell survival, with a critical role in inhibiting the opening of the mPTP) along with eNOS, PKC and glycogen synthase kinase-3β (GSK) leading to cardioprotection (Davidson *et al.*, 2006; Hausenloy *et al.*, 2004). Conversely, Akt has also been implicated in cellular injury and caspase-dependent programmed cell death with caspase-3 playing a pivotal role in the regulation of the cellular apoptosis in several models including stress-induced myocardial injury models in rats (Hussain *et al*., 2014; Baines *et al*., 2005; Fulda *et al.,* 2010; Sakamaki and Satou, 2009).

Given the important findings that artemisinin has cardioprotective and anti-hypertrophic properties, the current study aims to examine the intracellular signalling pathways associated with artemisinin-mediated cardioprotection in the isolated rat hearts and adult primary ventricular myocyte models of I/R injury.

4.2 MATERIALS AND METHODS

Chemicals: Artemisinin, Wortmannin, Rapamycin were purchased from Tocris (Bristol, UK) and dissolved in DMSO and stored at -20 °C. MTT was purchased from Sigma (Poole, UK). Cleaved Caspase-3 (Asp175) Antibody (Alexa Fluor® 488 Conjugate), p-AKT $_{(Ser473)}$, total AKT, p-p70S6 Kinase $_{(Thr389),}$ total p70s6k, p-BAD $_{(Ser136,}$ total-BAD antibodies and secondary IgG HRP conjugated rabbit monoclonal antibodies were purchased from New England Biolabs (Hertfordshire, UK). SuperSignal West Femto®

enhanced chemoluminescent substrates was purchased from Fischer Scientific (Loughborough, UK)

Adult male Sprague Dawley rats (350-400g) were obtained from Charles River (Margate, UK). Animals received humane care in accordance with the guidance on the operation of the animals (Scientific Procedures Act 1986). The study was carried out upon obtaining ethical approval from Coventry University Research ethics committee which was regularly assessed throughout the project.

4.2.1 Isolated Perfused Heart Model

Following sacrifice by cervical dislocation, the hearts were rapidly excised and placed in ice cold KH solution(118.5 mM NaCl, 25 mM $NaHCO_3$, 4.8mM KCl, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 12 mM Glucose, 1.7mM $CaCl_2.2H_2O$) at <4 ^oC as described previously (Hussain *et al*., 2014).

The aortic arch was removed, allowing cannulation of the aorta and subsequent retrograde perfusion with KH buffer, saturated with 95% O_2, 5% CO_2, maintained at a temperature of 37 ± 0.5^oC and pH 7.4 using a water-jacketed heat exchange coil.

The left atrium was removed and a latex balloon inserted and inflated to a constant diastolic pressure of 8-10mmHg. This allows LVDP to be measured. A physiological pressure transducer was connected to a bridge amp and a power lab (AD Instruments Ltd, Chalgrove, UK) which allows the LVDP, HR using ECG leads and CF to be measured at regular intervals. The perfusate was also collected at those intervals.

During ischaemia/reperfusion studies the isolated hearts were allowed to stabilise for 20 minutes followed by 35 minutes ischaemia and 120 minutes reperfusion. Upon subjecting the hearts to 20 minutes of stabilisation, the anterior descending left coronary artery is ligated to induce regional ischaemia. This was performed using a hooked 6-0 silk surgical suture and forceps and piercing the heart under the left coronary artery to form a snare with the thread. The thread was passed through a pipette tip and the snare tightened to initiate ischaemia. At the onset of reperfusion, the flow of KH buffer was reintroduced via the removal of the pipette tip thus releasing the snare. Reperfusion was conducted for 120 minutes (Figure 33).

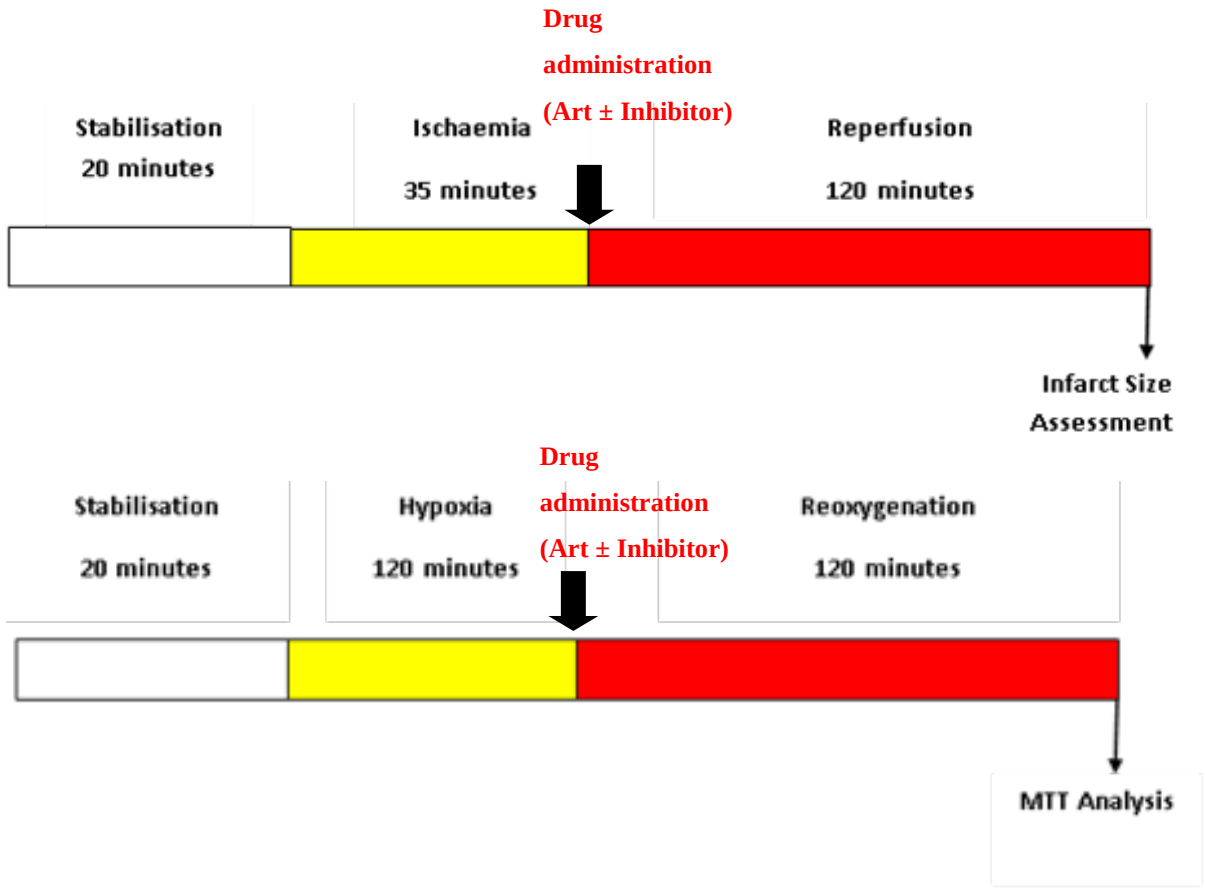


***Figure 33**. Treatment protocol for infarct size assessment studies and ventricular myocytes studies respectively.*

4.2.1.1 Treatment protocol for the Isolated rat heart model

The isolated hearts were allowed to stabilise for 20 minutes followed by 35 minutes of ischaemia and 120 minutes of reperfusion. At the onset of reperfusion, artemisinin EC_{80} at 4.3µM was administered throughout the experiment with the inhibitors of PI3k-Akt and p70S6K. Hearts were randomly assigned to different drug treatments. One group was treated with artemisinin (4.3µM) ± wortmannin (0.1µM) or with wortmannin (0.1µM) alone, which was administered throughout the period of reperfusion. Another group was treated with artemisinin (4.3µM) ± rapamycin (0.1µM) or with rapamycin alone (0.1µM) administered throughout reperfusion. Figure 33 illustrates an outline of the experimental protocol. No effects where seen in haemodynamics with the drug treated hearts when compared to control hearts.

Upon completing the experiment the left coronary artery re-ligated in preparation for staining the heart with 1ml of 0.2% Evans blue in saline, allowing differentiation between viable and tissue at risk. After staining, the hearts were weighed and stored at -20^{o}C for later analysis. The hearts were then cut transversely into slices approximately 2 mm thick and incubated at 37°C in 1% TTC solution in phosphate buffer for 10–12 stminutes and fixed in 10% formaldehyde for at least 4 hours to enhance the staining prior to analysis.

Thereafter, the heart slices were removed from formaldehyde and placed between two Perspex sheets which were compressed with bulldog clips. The heart slices were traced onto acetate film using different coloured markers to differentiate between the viable, at risk and infarct tissue. The at risk tissue stained red and the infarct tissue appeared pale.

The acetate film was scanned into a computer to allow calculation of Infarct to risk ratio from the differentiated tissues traced. Areas of viable, at risk and infarct tissue were measured using the Image Tool program as developed by the University of Texas Health Science Centre at San Antonio, Version 8.1 (UTHSCSA).

4.2.2 Adult Rat Ventricular Myocytes Isolation

Ventricular rat cardiomyocytes were isolated from Adult male Sprague Dawley rats (350-400g) by enzymatic dissociation method (Maddock *et al*., 2002; Hussain *et al*., 2014).

Following digestion as decribed in detail in chapter 2, the isolated myocytes were incubated in RB for MTT, caspase 3 and FACS analysis (at 37 ^{o}C, 5% CO_2 for 24 hours before being used (Maddock *et al*., 2002)

4.2.2.1 Treatment protocol for the isolated ventricular rat heart model

The RB suspended myocytes were then incubated in a hypoxic chamber, Galaxy 48R (New Brunswick) for 2 hours with atmosphere 5% CO_2 95% N_2 at 37^{o}C and the pellet resuspended in RB. The myocytes were then assigned to the different treatment groups: artemisinin (4.3µM), artemisinin (4.3µM) ± inhibitor of PI3K (wortmannin) (0.1µM), wortmannin (0.1µM), artemisinin (4.3µM) ± inhibitor of mTOR/p70S6K (rapamycin) (0.1µM), rapamycin (0.1µM). The myocytes then underwent reoxygenation for 2 hours. Upon completing reoxygenation the cells were then assessed either for cellular viability using MTT or flow cytometric analysis for p-BAD $_{(Ser136)}$ or cleaved caspase-3 activity as described below.

4.2.3 Quantitative analysis of cleaved caspase-3 activity and BAD $_{(Ser136)}$ by FACS analysis

Isoated cardiomyocytes were resuspended in PBS and fixed with 3% formaldehyde for 10 minutes at room temperature washed in incubation buffer (0.5% BSA in PBS as described in detail previously. For p-BAD $_{(Ser136)}$ and T-BAD analysis, the samples were probed for 1 hour with either p-BAD $_{(Ser136)}$ or T-BAD rabbit monoclonal antibody diluted at 1:100 dilution in incubation buffer followed by incubation in Alexa Fluor® 488 goat anti-rabbit IgG antibody at

a dilution of 1:1000 for 1 hour. For the analysis of cleaved caspase-3 activity, the cells were incubated for 1 hour in cleaved caspase-3 $_{(Asp175)}$ secondary rabbit monoclonal antibody (Alexa Fluor ® 488 conjugate) (New England Biolabs, Hertfordshire,UK) diluted at 1:1000 . At the end of the incubation period, the cells were centrifuged and the supernatant was removed the cells resuspended in 500µl PBS and analysed using flow cytometer (Becton Dickinson, Oxford, UK) using the on the FL1 channel to count 10,000 events (Vermes *et al.*, 2002; Hussain *et al.*, 2014).

4.2.4 Cell viability assay based on MTT reductase activity

The cells were harvested as described previously and subjected to MTT assay to measure succinate dehydrogenase activity. 50µl of cells (containing 1 x 10^{-4} cells.ml^{-1}) were used per well and for contained 100µl of restoration buffer. Other wells were used for the different treatment groups which contained 50µl of cells and 50µl of drug treatment. Drugs used for this study were diluted with restoration buffer to a final concentration of 4.3µM in the artemisinin treated group, 0.1µM in wortmannin treated groups and 0.1µM in rapamycin treated groups similar to previous experiments which were administered at reoxygenation. 6 wells were used for each concentration of the experimental groups used. Cells were the incubated for 2 hours under hypoxic conditions. Drugs were administered at the start of reoxygenation and cells were incubated for an additional 2 hours with 20µl of MTT (5mg.ml^{-1} in PBS) which was added to all the wells and incubated in the dark at 37^{0}C. 100µl of lysis buffer (20% SDS in 50% dimethylformamide) was then added and cells incubated overnight at 37^{o} C. The absorbances of the plates were measured on a plate reader at 450nM (Thermo Scientific, UK).

4.2.5 Western Blotting

Following the protocol previously described in chapter 2, 60µg of the sample was loaded onto Biorad 4-15% Tris/Glycine precast gradient gels (Biorad, Hertfordhsire, UK). The gel was then attached to the Mini-PROTEAN 3 electrode assembly system (Biorad PowerPac 3000) and run at 130volts for 90 minutes (Figure 34).

Following electrophoresis, the proteins were transferred unto a PVDF membrane using the Biorad Midi Trans-Blot® Turbo™ Transfer pack using the Biorad trans blot at 25V, 1.3 A for 7 minutes when using two gels. The membrane was washed in TBST and incubated with blocking buffer (5% milk in TBST) for an hour. The membrane was washed 3 times in TBST and then incubated overnight on an orbital shaker at 4^{o}C with p-Akt $_{(Ser473}$ or p-p70S6K $_{(Thr 389)}$ rabbit monoclonal antibody at a dilution of 1:1000. After the overnight incubation, the

membrane was washed 3 times in TBST and then incubated in a 1:2000 dilution of Anti-rabbit antibody HRP linked IgG and HRP linked anti-biotin antibody Immunoblots were detected with the Super Signal West Femto (Fischer Scientific, Loughborough, UK) using the Biorad™ ChemiDoc imaging system (as shown in Figure 34).

Figure 34: Illustration of a typical western blot set up used to quantify protein. Avaiable online from < http://www.antibodies-online.com/images/news/WesternBlotSetup.jpg>

Band intensity was quantified using Quantity-One 1-D analysis software (Biorad, Hertfordshire, UK) for the p-Akt or p-p70s6k expressions Upon capturing the images of the relative changes in density, the blots were then stripped of the phospho-antibody (p-Akt or p-p70S6K) by boiling in water for 5 minutes and membranes re-probed for T-Akt or T-p70S6K by incubating in (5% milk in TBST) for an hour, then incubated overnight on an orbital shaker at 4^{o}C for Totals (T-Akt and T-p70s6k) at a dilution of 1:1000. After the overnight incubation, the membrane was washed 3 times in TBST and then incubated in a 1:2000 dilution of Anti-rabbit antibody HRP linked IgG and HRP linked anti-biotin antibody. Immunoblots were detected on the Biorad™ ChemiDoc imaging system as done earlier. The relative changes in phosphorylated proteins were normalised to T-Akt and T-p70S6K expression.

Statistical Analysis

The percentage of infarct/risk ratio, % of cell viability and the relative changes in phosphorylated proteins were all expressed as MEAN±SEM. Hearts treated with artemisinin, the different inhibitors and the control groups were tested for group differences in infarct size,

cellular viability and protein expression using the SPSS software package-One Way analysis of variance (Anova) with LSD post hoc test. Physiological parameters (Haemodynamics) were assessed using Two Way Anova. P values of $P<0.05$ were considered statistically significant.

4.3 RESULTS

4.3.1 Haemodynamics

There was no significant effect on the haemodynamic parameters with drug treated hearts as compared to control (data not shown).

4.3.2 Exclusion Criteria

We have excluded two rats from our experiment, one due to poor stabilization and the other due to very high flow rate which may be indicative of a tear in the aortic wall or may be an incompetent valve. Another rat was also excluded due to low viability of live cells following isolation (≤70%) in the cellular viability studies. All other groups have been used.

4.3.3 Artemisinin confers protection from I/R via activation of the PI3K cell survival pathway in the isolated perfused heart model

Administration of artemisinin (0-100μM) throughout reperfusion significantly ($P<0.01$-0.001) reduced infarct size by limiting myocardial ischaemia reperfusion injury as shown in chapter 3. EC_{80} which was calculated as 4.3μM (the most effective cardioprotective concentration) was used in subsequent experiments as was illustrated in Figure 20c.

To elucidate the mechanistic basis of artemisinin-induced protection of ischaemic reperfusion injury, we evaluated the role of the PI3K pathway. Artemisinin (at 4.3μM) which caused a significant ($P<0.001$) decrease in infarct size to risk ratio compared to the I/R control (38.0±2.5% vs.55.8±1.7% respectively, Figure 35). Wortmannin alone (at 0.1μM) had no effect on infarct size to risk ratio compared to control (51.9 ± 1.3% vs. 55.8 ± 1.7 % respectively, Figure 35).

However, co-administration of artemisinin (4.3μM) with wortmannin (at 0.1μM) significantly ($P<0.01$) reversed artemisinin-induced infarct sparing effects compared to artemisinin (4.3μM) alone (48.7±1.1% vs. 38.0 ±2.5% respectively, Figure 35).

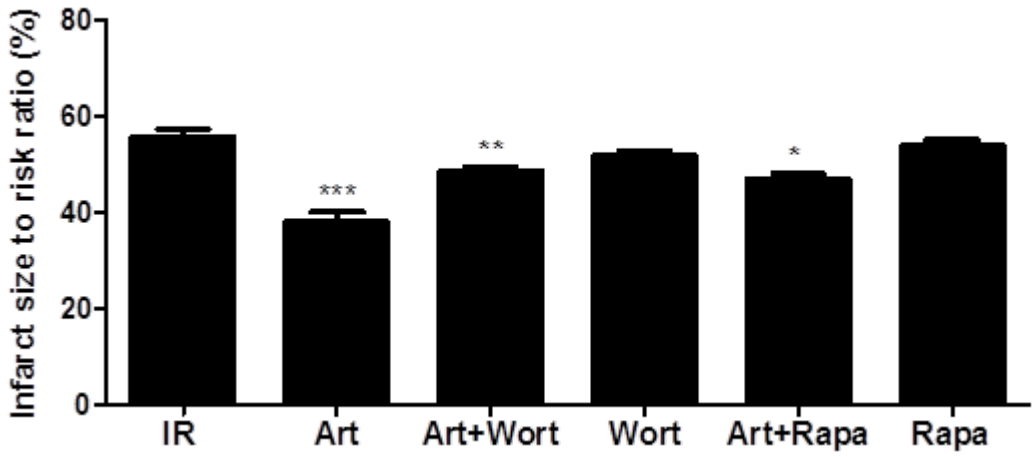


Figure 35. *Assessment of PI3K-Akt cell survival pathway in artemisinin mediated cardioprotection in isolated perfused heart model subjected to 35 minutes ischaemia followed by 120 minutes reperfusion. Artemisinin (Art) (4.3µM) (EC_{80}) was administered at reperfusion in the presence and absence of wortmannin (Wort) (PI3K inhibitor) (0.1µM) or rapamycin (Rapa) (mTOR inhibitor) (0.1µM) .Wortmannin (0.1µM) or rapamycin (0.1µM) was also administered alone at reperfusion and subjected to same experimental protocol. Results are shown as Mean±SEM. (***P<0.001 vs IR, **P<0.01 vs Art and *P<0.05 vs Art) (n=6-8).*

Rapamycin alone (at 0.1µM) did not have an effect on I/R compared to I/R control (53.9±1.6% vs 55.8 ± 1.7% respectively), but the co-administration of artemisinin (4.3µM) with rapamycin (0.1µM) upon reperfusion significantly (P<0.05) reversed the protective effects of artemisinin (47.0±1.4 % vs 38.0±2.5 % respectively, Figure 35).

4.3.4 Artemisinin improves the viability of cardiomyocytes subjected to hypoxia/ reoxygenation injury via PI3K cell survival pathway

The isolated cardiac myocytes were subjected to 2 hours of hypoxia and 2 hours of reoxygenation. Artemisinin (4.3µM) was administered throughout the reoxygenation period in the presence and absence of the PI3K inhibitor wortmannin (0.1µM).

2 hours of hypoxia followed by 2 hours of reoxygenation significantly (P<0.001) decreased the viability of the cells 29.3±6.1 % H/R vs. 100.0±0.0% respectively, Figure 36). Administration of artemisinin (4.3µM) throughout reoxygenation significantly improved cell viability compared with H/R group (66.5±6.3% vs. 29.3±6.1 % respectively, P<0.01, Figure 36).

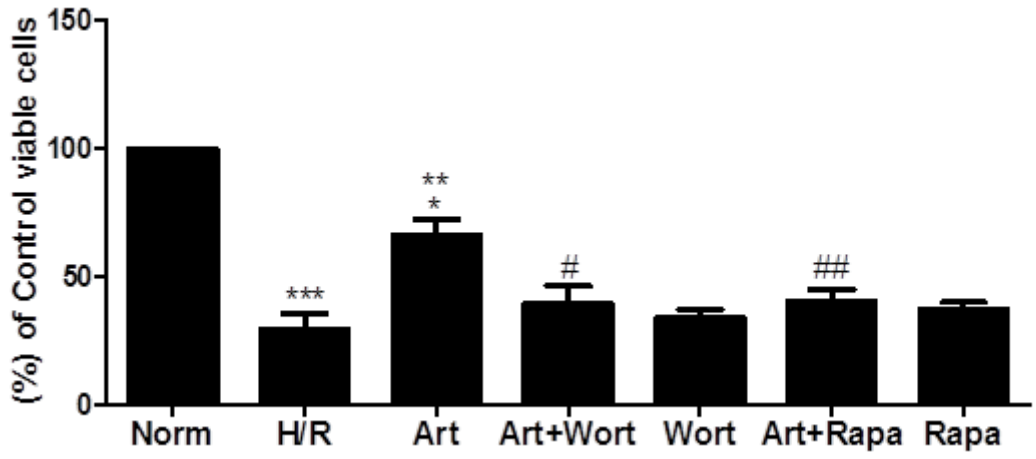


Figure 36**: % change in MTT reductase activity compared with control in isolated rat ventricular myocytes following hypoxia/reoxygenation protocol. For all drug treated groups except artemisinin (Art) (4.3μM) was administered at the start of reoxygenation with/without co-administering the inhibitors wortmannin (Wort) (PI3K inhibitor) (0.1μM) or rapamycin (Rapa) (mTOR inhibitor)(0.1μM). The inhibitors, wortmannin (0.1μM) or rapamycin (0.1μM) were also administered alone at reoxygenation and subjected to same experimental protocol. Values are mean ± SEM (P<0.001 vs Norm, *P<0.05 vs Norm, **P<0.01 vs H/R, #P<0.05 vs Art and ##P<0.01 vs Art) (n=6)*

Administration of wortmannin alone (0.1μM) above at reperfusion had no effect on cellular viability (33.7±3.8% vs. 29.3±6.1%, P>0.01 respectively, Figure 36). However, the co-administration of artemisinin (4.3μM) with wortmannin (0.1μM) significantly (P<0.05) reversed the viability afforded to the cells by artemisinin (39.5 ±7.6% vs. 66.5 ± 6.3% respectively, Figure 36).

Rapamycin alone (0.1μM) compared to H/R had a no significant effect on cellular viability (31.8±3.2 vs. 29.3±6.1% respectively, Figure 36). Co-treatment with the mammalian target of rapamycin (mTOR) inhibitor, rapamycin significantly (P<0.01) blocked the cytoprotective activity of artemisinin (4.3μM) (39.5±5.1% vs. 66.5±6.3% respectively, Figure 36).

4.3.5 Artemisinin decreases Cleaved Caspase-3 activity in cardiomyocytes subjected to hypoxia reoxygenation injury

To determine the cell signalling mechanisms via which artemisinin mediates its cytoprotective effects, isolated rat ventricular myocytes were subjected to 2 hours of hypoxia followed by 2 hours of reoxygenation. Cells at reoxygenation were treated with artemisinin (4.3μM) alone and in the presence/absence of PI3K inhibitor, wortmannin (0.1μM) or mTOR inhibitor, rapamycin (0.1μM) from which we assessed the levels of cleaved caspase-3 via flow cytometric analysis. Results from H/R group show a significant (P<0.001) increase in the levels

of cleaved caspase-3 activity compared to normoxic control (26.8±2.0% vs. 8.8±1.2% respectively, Figure 37).

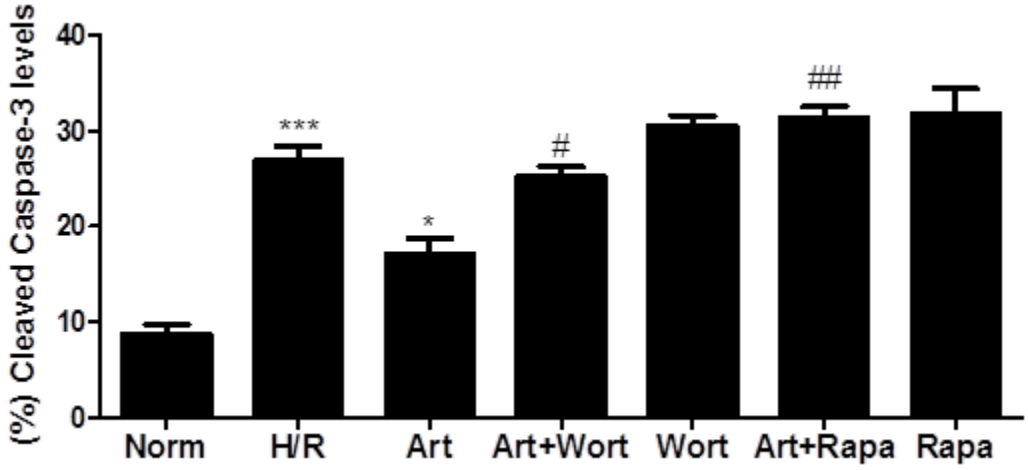


Figure 37**: % increase in Cleaved Caspase-3 activity compared to normoxia showing the effect administering Artemisinin (Art) (4.3μM) at the start of reoxygenation with/without co-administering inhibitors wortmannin (Wort) (PI3K inhibitor) (0.1μM) and rapamycin (Rapa) (mTOR inhibitor) (0.1μM) . The inhibitors, wortmannin (0.1μM) or rapamycin (0.1μM) were also administered alone at reoxygenation and subjected to same experimental protocol. Values are Mean±SEM (P<0.001 vs Norm, *P<0.05 vs H/R, # P<0.05 vs Art and ##P<0.01 vs Art)(n=4-6)*

The administration of artemisinin (4.3μM) at reoxygenation significantly (P<0.05) decreased cleaved caspase-3 activity when compared to H/R group (17.1±2.0% vs. 26.8±2.0% respectively, Figure 37). The treatment of the cells with 0.1μM wortmannin alone did not have an effect on cleaved caspase-3 activity compared to untreated time matched control (30.5±1.2% vs. 26.8±2.0% respectively, Figure 37). However, the anti-caspase 3 activity of artemisinin was significantly (P<0.05) reversed by co-administration of wortmannin (25.2±1.3% vs. 17.1±2.0% respectively, Figure 37). The treatment of cells with rapamcycin alone (0.1μM) also did not have an effect on cleaved caspase-3 activity compared to untreated time matched controls (31.3±1.5 vs 26.8±2.0% respectively, Figure 37). Whereas, anti-caspase 3 activity of artemisinin was significantly (P<0.001) reversed by co-treatment of artemisinin (4.3μM) with 0.1μM rapamycin (31.3±1.5% vs 17.1±2.0% respectively, Figure 37).

4.3.6 Artemisinin mediated cardioprotection involves upregulation of p-AKT (Ser473) in I/R treatment with artemisinin (4.3μM) in the presence and absence of wortmannin (0.1μM)

Isolated perfused hearts were subjected to 35 minutes of ischaemia and reperfused for 10 minute, where artemisinin (4.3μM) was perfused in the presence and absence of the PI3K

inhibitor, wortmannin. Administration of artemisinin (4.3µM) significantly (P<0.01) upregulated p-Akt $_{(Ser473)}$ expression compared with time-matched controls (129.4±15.9% vs. 26.8 ± 11.4% respectively, Figure 39a & 39b).

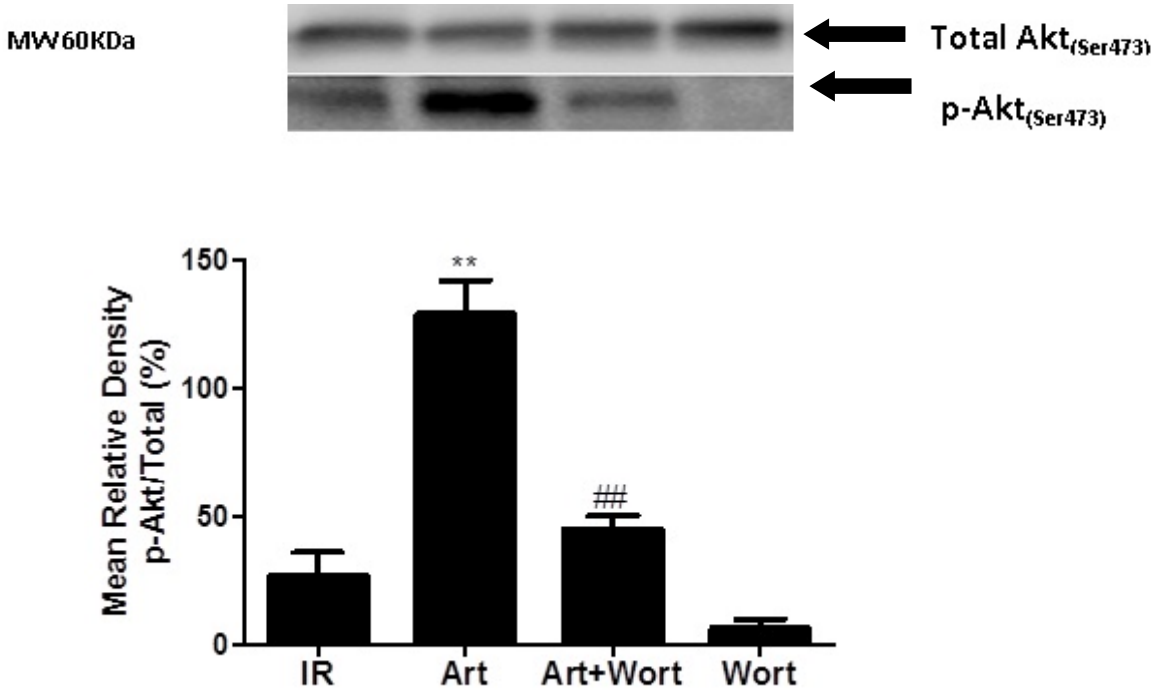


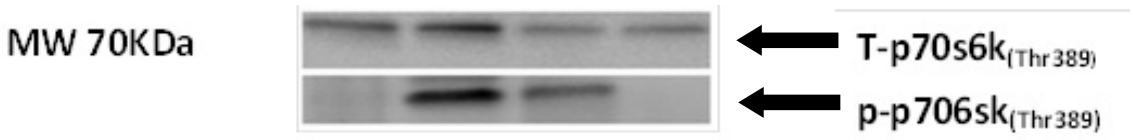
***Figure 38a**: A representative blot of p-Akt and Total Akt from isolated rat heart tissue in response to the treatment with Artemisinin (Art) (4.3µM), a combination of Art (4.3µM) and wortmannin (Wort) (0.1µM) (ie Art+Wort) and Wort (0.1µM) alone in the ischaemic reperfusion model.*

***Figure 38b**: Western blot demonstrating Phospho-Akt $_{(Ser473)}$ expression with Artemisinin (Art) 4.3µM and Wortmannin (Wort)(PI3K inhibitor)(0.1µM) which abolishes expression induced by Artemisinin. Wortmannin (0.1µM) was also administered alone at reperfusion and subjected to same experimental protocol as the Artemisinin treatment. (**P<0.01 vs IR and ##P<0.05 vs Art)(n=4).*

The treatment of the hearts with wortmannin (0.1µM) alone did not have any effect on the level of Phospho-Akt $_{(Ser 473)}$ compared to untreated time matched control (6.4±4.4% vs. 26.8±11.4% respectively, Figure 38b). However, co-treatment of artemisinin (4.3µM) with wortmannin (0.1µM) showed a significant (P<0.01) decrease in the level of Phospho-Akt $_{(Ser473)}$ compared to artemisinin (4.3µM) alone (45.3±6.2% vs. 129.4±15.9% respectively, Figure 38b).

4.3.7 Artemisinin mediated cardioprotection involves the upregulation of phosphorylated p70S6K $_{(Thr389)}$ in I/R treatment with artemisinin (4.3µM), rapamycin (0.1µM)

Hearts subjected to 20 minutes stabilisation, 35 minutes ischaemia and 120 minutes reperfusion with artemisinin and (4.3µM) and rapamycin (0.1µM) as previousy described. Proteins were extracted from ventricular tissue samples and were loaded unto gradient gels. Following electrophoresis, immunoblots of the different treatments and controls were detected and the relative changes in phosphorylated proteins following treatment were quantified. Artemisinin (4.3µM) showed a significant ($P<0.001$) upregulation in the level of phospho- p70S6K $_{(Ser389)}$ compared with time matched controls (76.0± 5.5% vs. 10.0±2.0% respectively, Figure 39). Treatment with rapamycin (0.1µM) alone at reperfusion had no effect on phospho-p70S6K $_{(Thr389)}$ levels compared to IR control (4.5±3.2% vs 10.0±2.0% respectively, $P<0.05$, Figure 39). However, co-treatment of artemisinin (4.3µM) with rapamycin (0.1µM) showed a significant ($P<0.05$) decrease in the level of phosphorylated p70S6K $_{(Ser389)}$ compared to artemisinin (4.3µM) alone (49.9±7.0% vs. 76.0±5.5% respectively, Figure 39).



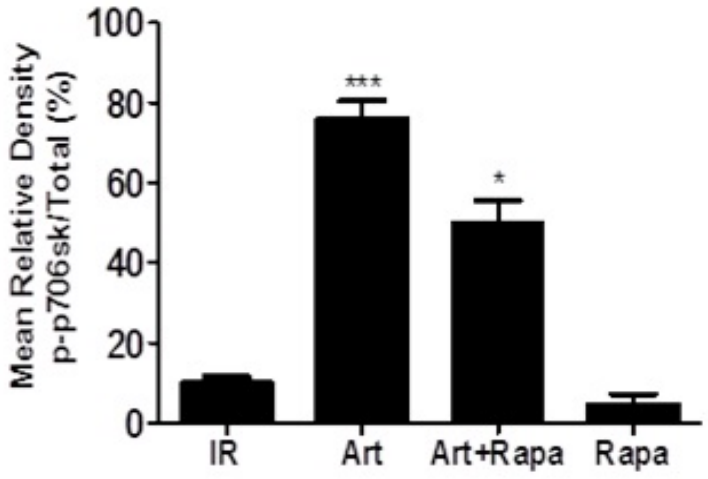


***Figure 39a**: Above is the representative blot of of p-p70S6K and T-p70S6K from isolated rat heart tissue in response to the treatment with Artemisinin (Art) (4.3μM) and rapamycin (rapa)(0.1μM) in combination (artemisinin and rapamycin) (Art+rapa) and alone in the ischaemic reperfusion model.*

Figure 39b**: Western blot demonstrating Phospho-p70s6k (Thr389) expression with Artemisinin (Art) 4.3μM and rapamycin (Rapa) (mTOR inhibitor)(0.1μM) which abolishes expression induced by Artemisinin. Rapamycin (0.1μM) was also administered alone, at reperfusion and subjected to same experimental protocol. (P<0.001 vs IR and *P<0.05 vs Art)(n=4).*

4.3.8 Artemisinin mediated cardioprotection involves upregulation of p-BAD (Ser136)

Flow cytometric analysis was used to determine p-BAD (Ser136) in cardiomyocytes subjected to hypoxia reoxygenation injury where the effects of artemisinin (4.3μM) and wortmannin (0.1μM) were investigated alone and in combination. Hypoxia/Reoxygenation resulted in a significant (P<0.001) decrease in p-BAD (Ser136) compared with the normoxic group (62.0± 7.4% vs. 100.0±0.0% respectively, Figure 40). The treatment of the cells with artemisinin (4.3μM) caused a significant (P<0.01) increase in p-BAD (Ser136) levels compared to H/R control (106.9±8.8% vs. 62.0±7.4% respectively, Figure 40) whereas co-incubating artemisinin (4.3μM) with wortmannin (0.1μM) significantly (P<0.05) decreased the artemisinin dependent increase in p-BAD (Ser136) levels (77.3±2.1% vs. 106.9±8.8% respectively, Figure 40). Whereas administering wortmannin (0.1μM) throughout the reoxygenation phase had no significant effect on phospho-BAD (Ser136) levels compared with H/R control group (59.9±2.0% vs. 62.0±7.4%, respectively, Figure 40).

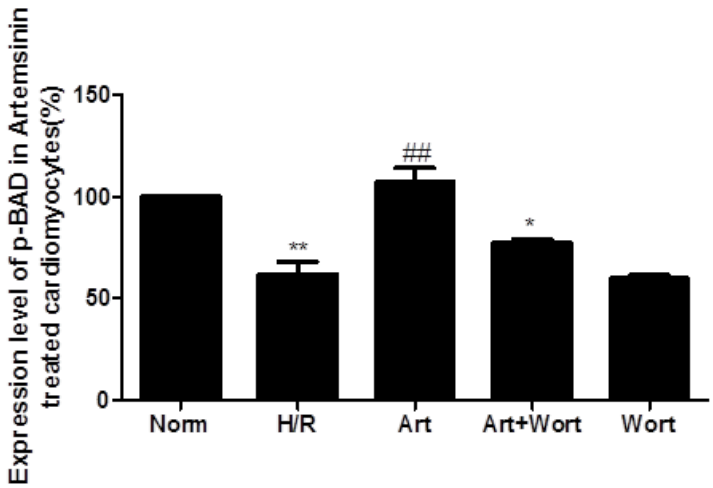


Figure 40*: The assessment of phosphorylated BAD expression as analysed using flow cytometric analysis upon treatment with Artemisinin (Art) (4.3µM) at reoxygenation following the hypoxia/reoxygenation protocol in the presence and absence of Wortmannin (Wort)(PI3K inhibitor)(0.1µM0.vValues are expressed as MEAN±SEM (**P<0.01 vs Norm,##P<0.01 vs H/R,*P<0.01 vs Art)(n=6-8).*

4.4 DISCUSSION

It is well established that artemisinin possesses anti-parasitic, anti-cancer, anti-inflammatory and anti-viral properties (Ding *et al*., 2014; Ho *et al*., 2014; Mavoko e*t al*., 2013; Maude *et al*., 2010; Santos *et al*., 2002). There is some evidence in a myocardial model of I/R injury using Wistar rats suggesting artemisinin alleviates myocardial ultrastructure injury and possesses some cardioprotective effects that can salvage cardiac myocytes by limiting infarct size development (Sun *et al*., 2007) at 10µM and 100µM concentrations investigated, however, the signalling pathways mediating this protection were not established.

In chapter 3, we have we investigated the concentration-dependent (0-100µM) effect of artemisinin on infarct size to risk ratio and EC_{80} (4.3µM) concentration, was used in this study as it was identified as the most effective concentration. This concentration of artemisinin was used to identify the intracellular signalling associated with artemisin and to further enhance the understanding on mechanisms by which artemisinin is cardioprotective in the isolated perfused rat heart model, isolated cardiomyocytes subjected to MTT, cleaved caspase 3 activity, FACS analysis and Western blotting.

Previous studies have shown several cellular injuries occur as a result of drug induced cardiotoxicity which is a serious drawback amongst the most effective drugs ever developed

(Simanek *et al.,* 2009). The heart is an important off target organ of drug toxicity and an imbalance between supply and demand within an ischaemic organ in experimental studies have been shown to cause profound tissue hypoxia or may lead to microvascular dysfunction (Eltzschig and Eckle 2011). In this study we investigated the effect of artemisinin which we have shown to alleviate myocardial I/R injury in a dose dependent manner while particulary focusing on the celluar signalling mediating this protection. Ischaemia and reperfusion injury have been shown to elicit tissue injury which contributes to a number of clinical manifestations including CHD, myocardial infarction and other pathologies which also remains a leading cause of death worldwide (Eltzschig and Eckle 2011, Kloner *et al.,* 2004; Hausenloy and Yellon 2013 and Ferdinandy *et al*., 2007). The ability of artemisinin to alleviate the simulated I/R injury by attenuating and preventing cellular damage proves artemisinin is an agent with great potential, given the potential promise from bench side to bedside.

In establishing artemisinin's association with PI3K-Akt cell survival pathway, we administered artemisinin (4.3µM) at the onset of reperfusion which significantly decreased myocardial infarct size development in a wortmannin-sensitive manner. Wortmannin is a potent, selective, cell-permeable and irreversible inhibitor of PI3K (Cheng *et al*., 2005). The PI3K–Akt pathway has been shown to participate in several cellular processes by phosphorylating a diverse array of substrates, including apoptotic proteins (BAD, BAX, BIM, p53 and caspases), eNOS and PKC (Li and Sato 2011, Madrid *et al.*, 2000 and Yang *et al.*, 2009). We also investigated the downstream targets of Akt such as BAD and p70S6K. Administration of rapamycin, an mTOR selective inhibitor in combination with artemisinin (4.3µM), the previously observed cytoprotective effects of artemisinin were abolished implicating p70S6K in artemisinin mediated protection.

It is well established that the activation of PI3K-Akt-p70S6K cell survival pathway is associated with the activation of pro-survival factors (via agents such as HMG-Co-A reductase inhibitors and G-protein-coupled receptor ligands, bradykinin and insulin-like growth factor-1) and inhibition of pro-apoptotic factors (such as-BAX, BIM and p53 and kinases implicated in I/R injury such as p38 and JNK MAPK, PKA, Rho kinase and JAK-STAT) (Li and Sato, 2001; Al khouri *et al.*, 2005; Hausenloy and Yellon, 2004). In the isolated rat heart model, infarct size was calculated in the different treatment groups and the physiological parameters monitored with the different treatments. Expression levels of phosphorylated proteins associated to the PI3K-Akt pathway and cardiomyocyte cell viability assessment were also

investigated and compared. To our knowledge, this is the first study to show that artemisinin confers protection to the myocardium following I/R in ex vivo model of myocardial I/R and the associated cell signalling pathway of artemisinin-mediated cardioprotection. However, Gu et al. (2012) have shown artemisinin can inhibit ventricular remodelling and cardiac and improve cardiac function possibly by the interruption of the NF-kB pathway.

We were able to show the involvement of the PI3K-Akt-BAD-p70s6K in both the isolated perfused heart model and isolated ventricular myocyte model. The co-treatment of artemisinin (4.3µM) with wortmannin (0.1µM) reversed the upregulation of p-Akt while rapamycin (0.1µM) reversed the expression of p70S6K thus confirming the involvement of PI3K-Akt-p70S6K cell survival pathway in artemisinin's cardioprotection. Hausenloy and Yellon (2004) have showed signalling through PI3-Akt-BAD pathway confers protection against I/R injury, through the activation of the serine–threonine kinase, Akt (Hausenloy and Yellon 2004) thus supporting our findings. Sun *et al.* (2013) also reported p-Akt in the mitochondria is responsible for inhibiting the release of cytochrome c as well as suppresses the activation of caspase-3 thereby limiting the number of cells committed to apoptosis.

Pharmacological agents such as resveratrol have been shown to utilise the same pathway by inhibiting the PI3K-Akt-p70S6K cell survival pathway in the human chronic myeloid leukemia K562 cell line (Sui *et al.*, 2014). The PI3K/Akt signalling pathway is critical in cell proliferation, differentiation and survival and in the study by Sui et al. (2014) resveratrol was shown to inhibit proliferation and induce apoptosis in various types of cancer cell. Furthermore, studies in cardiomyocytes subjected to I/R injury showed activation of the PI3K-Akt-p70S6K and its downstream targets such as eNOS, BAD, GSK-3β, mTOR and so on conferred protection upon administering insulin to rat heart subjected to myocardial injury and oxidative stress thus inhibiting apoptosis and improving cardiac function (Yao *et al.*, 2014).

Cleaved caspase-3, being a pivitol effector caspase in apoptotic signalling has been associated with I/R and cellular damage leading to apoptosis (Sakamaki and Satou 2009; Lu and Chen 2011). Studies have shown ROS induced stress is responsible for activating caspase-3 (Yuan *et al*., 2011). ROS induces mitochondrial oxidative stress which causes the outer mitochondrial membrane to rupture and release cytochrome c into the cytosol forming complexes known as apoptosomes which are cleaved into caspase-9 that activates caspase-3 eventually causing myocardial cell death (Banerjee *et al*., 2008; Yuan *et al*., 2011 Montaigne *et al*., 2012; Cui *et al*., 2012; Dai *et al*., 2014).

Artemisinin (4.3µM) administered at reoxygenation resulted in a significant decrease in hypoxic injury and in the levels of activated cleaved caspase-3 suggesting the cytoprotective role of artemisinin in ventricular myocytes is caspase dependent. Artemisinin's anti-caspase-3 activity was reversed by co-treatment with wortmannin (0.1µM) suggesting artemisinin's protection in the myocardium to be PI3K dependent. This thereby allowed us to extrapolate the existence of a novel link between PI3-Akt pathway and the anti-caspase-3 activity in artemisinin's discerned effect. Previous studies have described Akt as a critical target in suppressing apoptosis in a PI3K dependent cell survival pathway (Sun *et al.,* 2013; Abel and Doenst, 2011).

Improvement in myocyte viability was also observed upon administering artemisinin (4.3µM) at reperfusion following H/R, showing its capacity to salvage H/R induced injuries in adult ventricular myocytes. Aremisinin treated cells presented an increase in MTT reductase activity which was reversed with administering wortmannin (0.1µM).

Hausenloy and Yellon (2004) have shown the Reperfusion Injury Salvage Kinase (RISK) pathway to confer powerful cardioprotective responses which incorporates the anti-apoptotic pro-survival kinase signalling cascades, phosphatidyl-inositol-3-kinase (PI3K)–Akt and the p42/p44 extra-cellular signal-regulated kinases (Erk 1/2), both implicated in cellular survival. Our results have shown the up-regulation of pro-survival kinase PI3-Akt which have been confirmed by our western blot analysis to be implicated in the cytoprotection against reperfusion-induced injury in this study.

PI3K-Akt pathway has also been shown to protect against stress induced apoptotis (Kang *et al.,* 2010; Faghiri and Bazan 2010). Activation of Akt has been associated with cell survival and phosphorylation of BAD at $_{Ser136}$ (Datta *et al.,* 1997; del Peso *et al.,* 1997). Our results support this finding by showing an upregulation of p-BAD as well as Akt. BAD at Ser136 is one of the serine sites of phosphorylation which is responsible for neutralizing BAD's apoptotic function (Harada *et al.,* 2001). BAD ($_{Ser136)}$ is also the preferred target substrate for Akt and p70S6 kinases in the PI3K signalling pathway (Blume-Jensen *et al.,* 1998; Eves *et al.,* 1998 and Harada *et al.,* 2001). Our results suggest the involvement of BAD $_{(Ser136)}$ in artemisinin's cytoprotection which has been linked with the cell survival pathway PI3-Akt. Studies describe the translocation of BAD into the mitochondria which is usually accompanied by the release of cytochrome c which in turn initiates the cell death machinery initiating apoptosis (Mehic 2012). It is therefore our understanding that Akt which has been previously reported to be

translocated into the mitochondria where it modulates cellular death or survival to be the major player involved in this cellular survival (Yang *et al*., 2009; Abel and Doesnt 2011).

Our results also show artemisinin facilitates cell survival following I/R and H/R induced injuries by activating p70S6K, which is a functional target of Akt. Jonassen *et al*., 2004 has previously implicated p70S6K as a functional target of insulin activated Akt cell-survival signalling in their human cardiac derived Girardi model of I/R injury. Park *et al*. (2014) has also implicated protection from I/R injury by administering Cordycepin (3′-deoxyadenosine) isolated from *Cordyceps militaris* in adult Sprague Dawley rats via the activation of Akt/GSK-3β/p70S6K cell signaling pathway. Cordycepin like artemisinin has been shown to exhibit many pharmacological functions including anticancer, anti-inflammatory, and antioxidant activities and is speculated to be an attractive therapeutic agent against I/Rinjury and other cardiomyopathies (Park *et al*., 2014). Brevik *et al.* (2015) has similarly with intermittent exogenous B-type natriuretic peptide during early reperfusion (intermittent 3×30 seconds infusion of BNP peptide in early postconditioning) confers protection via PI3K/Akt/p70s6k dependent signalling suggesting the pivotal role of in cardioprotective signalling. Therefore the phosphorylation of p70s6k and BAD in response to Akt activation by administering artemisinin throughout reperfusion suggestively identifies artemisinin as a putative target in the induction of artemisinin-induced cardioprotection.

This study established the involvement of the anti-apoptotic pro-survival kinase signalling cascades, PI3K-Akt-BAD/p70S6K as being involved in artemisinin mediated cellular survival. Artemisinin was not only able to salvage the myocardium at risk, as observed by a reduction in infarct size but it also increased cellular viability and salvaged cells marked for apoptosis. Wortmannin and rapamycin inhibited the cardioprotective effects of artemisinin whereas no cardioprotective effect was observed indicating a role of PI3K-Akt/p70S6K in artemisinin mediated protection

To our knowledge this is the first study to associate artemisinin's cardioprotection to PI3K-Akt-BAD/p70S6K and anti-caspase-3 pathways. It has shown the cardioprotective artemisinin when administered at reperfusion salvages damaged tissue. This is supported by several studies that have linked PI3K pathway to be important in cardioprotection such as Hausenloy &Yellon (2004) who showed that the activation of PI3K-Akt survival kinase pathway constitutes a common survival pathway resulting in a cardioprotective response during reperfusion (Shneyvays *et al.*, 2005,Hussain *et al.*, 2014 and Sun *et al*., 2013)**.** The results from

the present study demonstrated treatment with artemisinin (4.3µM) significantly reduces infarct size and improves cellular viability when administered at reperfusion. Co-administering artemisinin with wortmannin or rapamycin abolished cardioprotection earlier observed by artemisinin alone. This thus shows the involvement of the recruitment of PI3K-Akt-BAD/p70S6K pathway by artemisinin at reperfusion (shown in Figure 41).

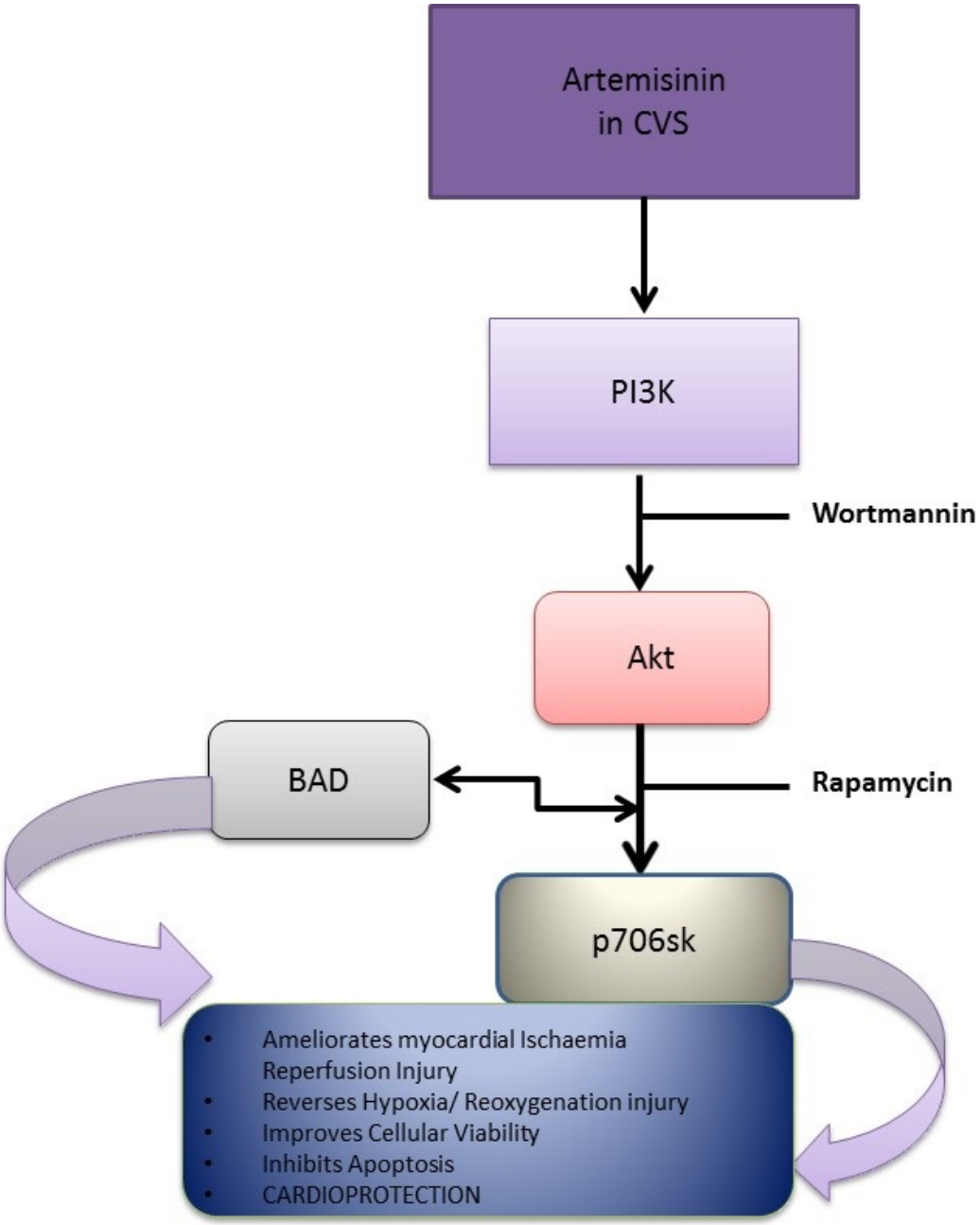


Figure 41*: Hypothetical schematic representation of the Artemisinin mediated cardioprotection in isolated cardiomyocytes and isolated perfused heart subjected to H/R injury and I/R respectively via the activation of the pro-survival signalling pathway PI3K-Akt kinase cascade and their targets BAD and p70S6K. PI3K-Akt is an important part of the RISK pathways identified by Hausenloy and Yellon (2003). Administering Artemisinin*

(4.3μM) at reperfusion has is suggested to initiate cardioprotection by protecting against reperfusion-induced cell death. The scheme portrays the important anti-apoptotic mechanisms that have been implicated in mediating cellular survival associated with the recruitment of these kinase cascades via the PI3K-Akt cascade shown to reverse the activation of caspases and proapototic protein BAD via the p70S6K upon administering Artemisinin in different settings of I/R and H/R.

4.5 CONCLUSION

This is the first study to show that artemisinin limits ischaemia reperfusion injury in the isolated rat heart and in cardiac myocytes via the recruitment of the PI3K-AKT-BAD/p70S6K cell survival pathway and may offer novel strategies for cardioprotection in a clinical setting.

Chapter 5

5 ARTEMISININ ATTENUATES MYOCARDIAL ISCHAEMIA REPERFUSION INJURY VIA RECRUITMENT OF THE NITRIC OXIDE CELL SURVIVAL PATHWAY

5.1 INTRODUCTION

The principal mechanisms for I/R injury involve ROS production and oxidative stress, with the major organs affected by this injury being the heart and lungs (Kozak 2007). During ischaemia, the vasculature of the heart becomes dysfunctional, as a result of coronary artery occlusion leading to an imbalance between myocardial oxygen supply and demand (Heusch 2008). This results in an ischemic cascade that releases cellular metabolic wastes that make the heart more vulnerable leading to ischaemic injury (Kozak 2007).

Reperfusion injury which also is an unavoidable clinical manifestation of myocardial injury has been shown to be associated with oxygen-derived free radicals mostly responsible for the observed injury (Annapurna *et al*., 2013). Drugs such as anthracyclines have been proven to have a double edged sword effect despite its effectiveness in treating cancers, it is shown to cause cardiomyopathy that could lead to more severe conditions or even death (Takemura and Fujiwara 2007).

This occurs so frequently that it has spurred research so much towards developing strategies to limit ROS induced injuries. The mechanisms thought to be responsible for the observed cardiotoxic effects with certain drug treatments are oxidative stress, disruption of calcium homeostasis and mitochondrial dysfunction (Minotti *et al*., 2004).

Excess productions of free radicals from either endogenous or exogenous sources have been shown to play significant roles in tissue damage and disease (Pharm-Huy *et al.,* 2008). There are also many substantial evidence from experimental studies that have associated oxidative stress and ischaemic heart disease (Bedard and Krause 2007; Rivera *et al.,* 2010; Young and Woodside 2001; Zhang *et al*., 2012).

Clinical observations have shown that there is an enhanced neutrophil mobilisation that leads to the release of highly reactive free radicals in patients suffering from ischaemia reperfusion injury (Peake and Suzuki 2004). These free radicals are known to cause tissue damage and increased production of nitric oxide, which is an effective mediator that reverses tissue injury (Kozak 2007).

Nitric oxide signalling is believed to be a promising therapeutic approach against myocardial dysfunction (Zhang *et al*., 2012). Due to the versatile nature of nitric oxide, it reacts readily with other free radicals such as hydroxyl to form nitrite or with superoxide in the mitochondria to form peroxynitrite which decomposes to become hydroxyl radical and nitrogen dioxide, respectively (Kozak, 2007). This process is similar to oxidative stress that produces reactive oxygen species, popularly known as 'nitrosative stress' which constitutes the RNI such as nitric oxide, peroxynitrite and S-nitrosothiols (Palcher *et al.,* 2007). RNI have been shown to react with proteins, carbohydrates and lipids, thus resulting in alterations in the intracellular and intercellular homeostasis of cells which may possibly lead to cell death and regeneration (Rahman *et al*., 2012). These reactive species damage cells by inactivating the metabolic enzymes and damaging important cellular components which exacerbate diseases such as ischaemia, atherosclerosis, inflammatory diseases and cancer (Rahman *et al.,* 2012; Isenovic *et al.,* 2011).

Nitric oxide is produced by the catalytic action of NOS on L-arginine (Palcher *et al.,* 2007). NOS is primarily responsible for the conversion of L–arginine to nitric oxide free radical upon reaction with oxygen which is an NADPH dependent oxidation (Palcher *et al.,* 2007). nNos, iNOS, eNOS are the different isoforms of NOS present in the human myocardium and are activated in response to ischaemia/hypoxia (Kelly *et al*., 1996).

Extracellular signals, such as shear stress, stimuli from vascular endothelial growth factor (VEGF), estrogen, sphingosine 1-phosphate, bradykinin, and aldosterone has been shown to modulate eNOS nitric oxide generation with Akt specifically inducing phosphorylation of Ser-1177 whereas phosphorylation of Thr-495 has been reported to down-regulate nitric oxide generation thus leading to cardioprotection (Chen *et al*., 2008).

During ischaemia, nitric oxide has been shown to enter endothelial cells and upon reoxygenation, NOS is activated to produce high amounts of nitric oxide, thus producing cytotoxic effects (Palcher *et al*., 2007). The cytotoxicity attributed to nitric oxide is due to peroxynitrite, which is produced from the reaction between nitric oxide and superoxide anion (Palcher *et al*., 2007). Generating these free radicals may have effects ranging from subtle cellular signalling to more exaggerated oxidative injury that may result in committing cells to necrosis or apoptosis (Palcher *et al*., 2007).

However, in most studies, nitric oxide has emerged as a very important intracellular messenger which is responsible for regulating virtually every critical cellular function (Gutierrez *et al.,* 2009). Nitric oxide is extremely important in regulating the vascular tone of endothelial vessels, thus playing an equally important role in the regulation of blood pressure, cardiac remodelling and cellular survival in cardiac hypertrophy and heart failure (Moncada and Higgs 1993; Wang *et al*., 1993; Zhoa *et al*., 1995). Limiting the incidences of ischaemia is therefore of vital importance, given that ischaemic heart disease is a leading cause of death in the industrialized world (Ferdinandy *et al*., 2007).

NOS has been associated with both physiological and pathological processes in the body Drugs such as angiotensin-converting enzyme inhibitors and statins have the ability to restore or maintain endogenous production of nitric oxide in endothelial cells; this mechanism may explain part of their therapeutic efficiency (Palcher *et al*., 2007).

Others studies have demonstrated that the Akt mediates the activation of eNOS in endothelial cells in response to shear stress, leading to increased nitric oxide production (Dimmeler *et al.,* 1997). By inhibiting the PI3K/Akt pathway or mutation of $_{\text{Ser 1177}}$ on the eNOS protein this decreases the Akt-dependent phosphorylation of the eNOS *in vitro* in a Ca^{2+} independent Manner (Dimmeler *et al.,* 1997). This study confirms eNOS as a novel

Akt target and nitric oxide generation to be critical for vascular remodelling thus making its decreased bioavailability to mediate the pathophysiological changes in vascular morphology often associated with hypertension and atherosclerosis (Rudic *et al.,* 1998).

Artemisinin, a traditional herbal drug has also been shown to have anti-hypertrophic effect in response to aortic banding by alleviating myocardial ischaemia reperfusion injury and exerting an inhibitory effect on inducible nitric oxide synthase synthesis (Xiong *et al.,* 2010; Sun *et al*., 2007).

In previous chapters' we have shown, artemisinin to be cardioprotective against myocardial I/R injury via activation of PI3K/Akt cell survival pathway which is an established cytoprotective signalling pathways (Haunsenloy and Yellon 2007; Yao *et al*., 2014). Phosphorylation of Akt has been shown to promote nitric oxide synthesis which is an established inhibitor of mitogenic and hypertrophic activities in cells (Hussain *et al.,* 2009; Ye *et al.,* 2010). Studies have also administered N^G-Nitro-l-arginine methyl ester (L-NAME) treatment to chronically inhibit eNOS (Abu-Soud *et al.,* 2000).

In the past decade, significant advances have been made in identifying intracellular pathways involved in the mechanisms that influence cellular survival and death (Fulda *et al.,* 2010). In this study we therefore aimed to determine involvement of the PI3K/Akt/iNOS-eNOS cell survival pathway in Artemisinin mediated cardioprotection. The comparative importance of the NOS isoforms, eNOS and eNOS-derived NO, initiate a cascade of molecular events leading to the activation of iNOS which is well linked to cardioprotection (Bolli *et al*., 1998). Thus investigating the Nitric Oxide pathway using non selective inhibitors of Nitric Oxide pathway L-NAME and a selective inhibitor of iNOS, Aminoguanidine in artemisinin mediated cardioprotection is vital in this study.

5.1.1 Aim and objective

The aim of this study was to determine whether administration of artemisinin (4.3μM) at reperfusion will reduced infarct size in isolated heart model, via improving cell viability in isolated cardiomyocytes following hypoxia reoxygenation (H/R) injury and whether the protective effect of artemisinin will be blocked when administered in combination with

inhibitors, L-NAME (100µM, non-specific nitric oxide inhibitor) and aminoguanidine (100µM, specific iNOS inhibitor).

5.2 METHODOLOGY

5.2.1 Animals

Adult male Sprague Dawley rats (350-400g) were obtained from Charles River (Margate, UK). The care and use of animals were in accordance with the Guidance on the Operation of the Animals (Scientific Procedures Act 1986). The study was carried out upon obtaining ethical approval from Coventry University Research ethics committee which was regularly assessed throughout the project.

5.2.2 Preparation of drugs

Artemisinin, L-NAME) (Non-specific inhibitor of Nitric Oxide) and Aminoguanidine hydrochloride (Selective inducible nitric oxide inhibitor) both supplied from Sigma-Aldrich (Poole, UK) and dissolved in DMSO making sure the final concentration of DMSO was less than 0.02% as this does not affect haemodynamics or infarct size (unpublished paper) stored at -20 °C. MTT was purchased from Sigma (Poole, UK). Cleaved Caspase-3 (Asp175) Antibody (Alexa Fluor® 488 Conjugate), iNOS, phospho-eNOS (Ser1177), eNOS Horseradish peroxide (HRP) conjugated Rabbit monoclonal antibodies and antibiotin were purchased from New England Biolabs (Hertfordshire, UK). SuperSignal West Femto® enhanced chemoluminescent substrates were purchased from Pierce (UK). Nitric oxide Assay was purchased from Cell Biolabs Inc (San Diego, USA). HL-60 cells were obtained from ECACC.

5.2.3 Isolated perfused rat heart model

Following sacrifice by cervical dislocation, the hearts were rapidly excised and placed in ice cold KH solution (118.5 mM NaCl, 25 mM $NaHCO_3$, 4.8mM KCl, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 12 mM Glucose, 1.7mM $CaCl_2.2H_2O$) $<4\,^{o}C$ and pH 7.4 as described previously (Hussain *et al.*, 2014).

The aortic arch was removed, allowing cannulation of the aorta and subsequent retrograde perfusion with KH buffer, saturated with 95% O_2, 5% CO_2, maintained at a temperature of 37 ± 0.5^oC and pH 7.4 using a water-jacketed heat exchange coil.

The left atrium was then removed and a water-filled latex balloon was inserted into the left ventricle to a diastolic pressure of 8-10mmHg. This allows LVDP to be measured. A physiological pressure transducer was connected to a bridge amp and a power lab (AD Instruments Ltd, Chalgrove, UK) which allows the LVDP, HR using ECG leads. CF was measured at regular intervals by collecting the perfusate at regular intervals.

The experiment was conducted for 175 minutes in total. During I/R studies the isolated hearts were allowed to stabilise for 20 minutes followed by 35 minutes ischaemia and 120 minutes reperfusion. Details of technique in chapter 2.

Artemisinin 4.3µM was administered throughout reperfusion in the presence and absence of either the non-selective NOS inhibitor L-NAME (100µM) or inducible Nitric Oxide Synthases (iNOS inhibitor) aminoguanidine (100µM).

Upon completing the experiment the left coronary artery re-ligated in preparation for staining the heart with 1ml of 0.2% Evans blue in saline, allowing differentiation between viable and tissue at risk. After staining, the hearts were weighed and stored at -20^oC for later analysis. The hearts were then cut transversely into slices, approximately 2 mm thick and incubated at 37°C in 1% TTC solution in phosphate buffer for 10–12 minutes and fixed in 10% formaldehyde for at least 4 hours to enhance the staining prior to analysis.

The heart slices were then removed from formaldehyde and traced onto acetate film using different coloured markers to differentiate between the viable, at risk and infarct tissue. The acetate film was scanned into a computer to allow calculation of Infarct to risk ratio from the differentiated tissues traced. Areas of viable, at risk and infarct tissue were measured using the Image Tool program as developed by the University of Texas Health Science Centre at San Antonio, Version 8.1 (UTHSCSA).

5.2.4 Adult Rat Ventricular Myocytes Isolation

Adult ventricular rat myocytes were isolated from Sprague Dawley rats (350-400g) by enzymatic dissociation method (Maddock *et al.*, 2003, Hussain *et al*., 2014). Details of heart digestion with collagenase digestion buffer in chapter 2.

Following digestion, the hearts were removed from the apparatus and the atria were trimmed away and discarded. The ventricles were mechanically disrupted by passing the tissue through a large bore Pasteur pipette. The tissues were then incubated for 10 minutes in 25ml of digestion buffer in an orbital shaker and oxygenated with 95% O_2 and 5% CO_2. Thereafter, the suspension was passed through a nylon mesh with a pore size 400µm and centrifuged at 400rpm for 2 minutes. The supernatant was discarded and the pelleted cells were re-suspended in restoration buffer (RB) (in mM 116 NaCL , 5.4 KCL , 0.4 $MgSO_4$, 10 glucose ,20 taurine, 5 Pyruvate, 0.9 $NaHPO_4$, 5 Creatine , 2% BSA, 50µM $CaCl_2$ and 1% Penstrep pH 7.4 at 37^o C) where the calcium concentration was gradually brought to 1.25mM to avoid calcium overload. The isolated myocytes were incubated in RB (at 37^{o}C, 5% CO_2 for 24 hours before being used (Maddock *et al*., 2002)**.**

Figure 42. *An Image of isolated ventricular myocytes*

The cells were incubated in 15 mls of hypoxic buffer (12 mM KCL, 0.49 mM $MgCl_2$, 0.9 mM $CaCl_2$, 4 mM HEPES, 10 mM Deoxyglucose and 20 mM lactate and placed into a hypoxic chamber pre-heated at 37 °C. Following incubation, the myocytes were centrifuged at 500rpm for 5 minutes and the pellet was resuspended in restoration buffer. The myocytes were then assigned to the different treatment groups: artemisinin (4.3µM), artemisinin (4.3µM) + L-NAME (100µM), L-NAME (100µM), artemisinin (4.3µM) + aminoguanidine (100µM) and aminoguanidine (100µM). The myocytes then underwent

reoxygenation for 120 minutes. Upon completing reoxygenation the cells were then assessed either for cellular viability using MTT (Thiazolyl blue tetrazolium bromide) or flow cytometric analysis for iNOS, eNOS $_{(Ser\ 1177)}$ or cleaved caspase-3 activity as described below.

5.2.5 Cellular viability assay based on Thiazolyl blue tetrazolim bromide (MTT) reductase activity using isolated ventricular cardiomyocytes

The isolated myocytes were counted using a using a nucleo counter (Chemometec, Sartorius, Surrey, UK) and resuspended in RB to a density of 100,000 cells/ml. 1ml of the cells was pipetted to be used as normoxic control while the remaining cells were centrifuged and the pellet re-suspended in Esumi hypoxic buffer (in mM 137 NaCl ,12 KCL, 0.49 $MgCl_2$ 0.9,$CaCl_2$, 4 HEPES,20 Na lactate, 10 deoxy-D-glucose). The myocytes were then incubated in a hypoxic chamber, Galaxy 48R (New Brunswick) for 120 minutes with atmosphere 5% CO_2 95% N_2 at 37^{o}C.

Some wells were used as control which contained 100µl of restoration buffer. The remaining wells were randomly allocated to the different treatment groups which contained 50µl of cells and 50µl of drug treatment. Drugs used for this study were diluted with restoration buffer to a final concentration of 4.3µM in the artemisinin treated group, 100µM in L-NAME treated group and 100µM in aminoguanidine treated group. The drugs were administered at the start of reoxygenation and cells incubated for an additional 120 minutes with 20µl of MTT (MTT solution consisting of 5mg.ml^{-1} in PBS (10g for 10^{-4} cells/well) was added at reoxygenation except for the blanks which contains restoration buffer and MTT solution. The cells were then incubated in the dark at 37^{o}C for 120 minutes. Upon completing the 120 minutes reoxygenation, myocytes were subsequently lysed with 100µl of lysis buffer (20% SDS in 50% dimethylformamide) and incubated on an orbital shaker and incubated overnight at 37^o C. Calorimetric analysis of the plate was done to measure the fluorescence emission at 450nm (Thermo Scientific, UK) using a plate reader (Anthos 2001). The absorbance was measured for the different treatment groups. The effect of the artemisinin treatment was obtained by subtracting the absorbance from the control values.

Graphs were made using the mean absorbance of the drug treated group as a percentage of the mean absorbance of the control group.

5.2.6 Quantitative analysis for cleaved caspase-3, iNOS and eNOS (Ser 1177) using FACS analysis

Following the cardiomyocyte isolation, the myocytes were harvested and centrifuged at 1200 rpm for 2 minutes. The pellet was then resuspended in PBS and fixed with 3% formaldehyde for 10 minutes at room temperature. The cells were then put on ice for 1 minute before centrifuging at 1200 rpm for 2 minutes following aspiration of the supernatant. 250 µl of ice cold methanol (90%) was added and the samples incubated on ice for 30 minutes before being washed twice in incubation buffer (0.5% BSA in PBS) following a 10 minutes (at 37°C) incubation of the samples each time followed by centrifugation of the samples (at 1200rpm, 2 minutes). The antibody was prepared to 1:100 final dilution in incubation buffer for the analysis of cleaved caspase-3 activity or iNOS and eNOS.

5.2.7 Quantitative analysis of cleaved caspase-3 activity

Cleaved caspase-3 (Asp175) antibody (Alexa Fluor 488 conjugate) was used to detect the levels of activated caspase-3 in the different experimental groups. For the analysis of cleaved caspase-3 activity, the cells were incubated for 1 hour in cleaved caspase-3 (Asp175) secondary rabbit monoclonal antibody (Alexa Fluor ® 488 conjugate) (New England Biolabs, Hertfordshire,UK) diluted at 1:1000. At the end of the incubation period, the cells were centrifuged and the supernatant was removed the cells resuspended in 500µl PBS and analysed using flow cytometer (Becton Dickinson, Oxford, UK) using the on the FL1 channel to count 10,000 events (Vermes *et al.*, 2002; Hussain *et al.*, 2014).

5.2.8 Quantitative analysis of iNOS, p-eNOS (Ser 1177) and eNOS

To quantify iNOS levels, FACS analysis was used to assess the differential protein levels for iNOS and GAPDH. Following centrifugation, the harvested cells samples were probed for 1 hour with iNOS or GAPDH rabbit monoclonal antibody diluted at 1:100 dilution in incubation buffer followed by incubating in Alexa Fluor® 488 goat anti-rabbit IgG antibody at a dilution of 1:1000 for 1 hour.

For p-eNOS $_{(Ser\ 1177)}$ and T-eNOS the samples were also probed for 1 hour with p-eNOS and eNOS rabbit monoclonal antibody diluted at 1:100 dilution in incubation buffer followed by incubation in Alexa Fluor® 488 goat anti-rabbit IgG antibody at a dilution of 1:1000 for 1 hour.

At the end of the incubation period, the cells were centrifuged and the supernatant was removed the cells resuspended in 500µl PBS and analysed using flow cytometer (Becton Dickinson, Oxford, UK) on the FL1 channel to count 10,000 events (Vermes *et al*., 2002; Hussain *et al.*,2014).

5.2.9 Nitric oxide Assay using the OxiSelect™ In Vitro Nitric Oxide Calorimetric Assay

The OxiSelect™ is a colorimetric assay was used to measure the amount of nitrate and nitrite in the isolated cardiomyocytes treated with artemisinin (4.3µM). Assay was carried out according to manufacturer's instructions, details of which can be found in chapter 2.

5.2.9.1 Experimental protocol using the Nitric Oxide assay

Adult ventricular rat myocytes were isolated from Sprague Dawley rats by enzymatic dissociation method as in the previous protocols. The isolated myocytes were counted using a using a nucleo counter (Chemometec, Sartorius, Surrey, UK) and resuspended in restoration buffer (RB) to a density of 100,000 cells/ml.

5.2.9.2 Preparation of Samples and standards

Nitrite and nitrate standards were prepared by making a dilution series of concentration range 0-140µM from a standard 14mM of the nitrite and nitrate provided in the kit in restoration buffer. Potential interference was prevented by diluting the nitrate and nitrite standard in the same buffer as the samples. Triplicate wells were used for measuring the nitrate concentration.

5.2.9.3 Measurement of Total/Nitrate levels

Nitrate levels in the samples were measured by subtracting nitrite only from total nitrite and nitrate.

Nitrate = (Total nitrite + nitrate) - Nitrite only

50µL of nitrite standards, samples or blanks were added to a 96 well plate. Followed by 50µL of PBS and 50µL of Griess Reagent A were added to each well. The plate was then incubated for 10 minutes allowing the colour to develop. Absorbance was then read at 540nm on a microplate reader.

Concentration of nitrite was calculated by comparing sample absorbance to standards. Negative controls without nitrate were subtracted from reading. Each nitrite standard and sample was assayed in duplicate

5.2.9.4 Measurement of Total/Nitrate via Nitrate Reduction

To measure nitrate levels. 50µL of nitrate standard, samples or blanks to the 96 well plate. Drug treated cells were subjected to artemisinin (4.3µM) treatment. 50µL of the enzyme reaction mixture prepared according to manufacturer's instruction was then added to the wells containing the sample/nitrate and covered with foil.

The Plates were then incubated for 1 hour at room temperature on an orbital shaker. 50µL of Greiss Reagent A followed by Greiss Reagent B were added and the plate was then incubated for a further 10 minutes for colour development. Absorbance was then read at 540nm. The sample's absorbance is then calculated by comparing the Nitrate and standard curve and comparing our drug treatment to control.

Statistical Analysis

Results were expressed as Mean ±standard error of mean (SEM) for infarct/risk ratio, cell viability, cleaved caspase-3 analysis, iNOS, p-eNOS $_{(Ser\ 1177)}$ and nitric oxide assay. Infarct size was tested for group differences using one way analysis of variance (ANOVA) with LSD post hoc tests. P values of $P<0.05$ were considered statistically significant. Haemodynamics: LVDP, HR and CF were assessed for statistical difference using two way ANOVA at the different time points.

5.3 RESULTS

5.3.1 Exclusion Criteria

We have excluded three rats from our experiment; two due to low viability of live cells following isolation (≤70%) and the other due to a rip in the ventricular wall while tightening the snare during ischaemia. All other groups have been included.

5.3.2 Haemodynamics data for artemisinin treatments and inhibitors (L-NAME and aminoguanidine)

There was no significant effect on the different parameters when comparing the different treatment groups.

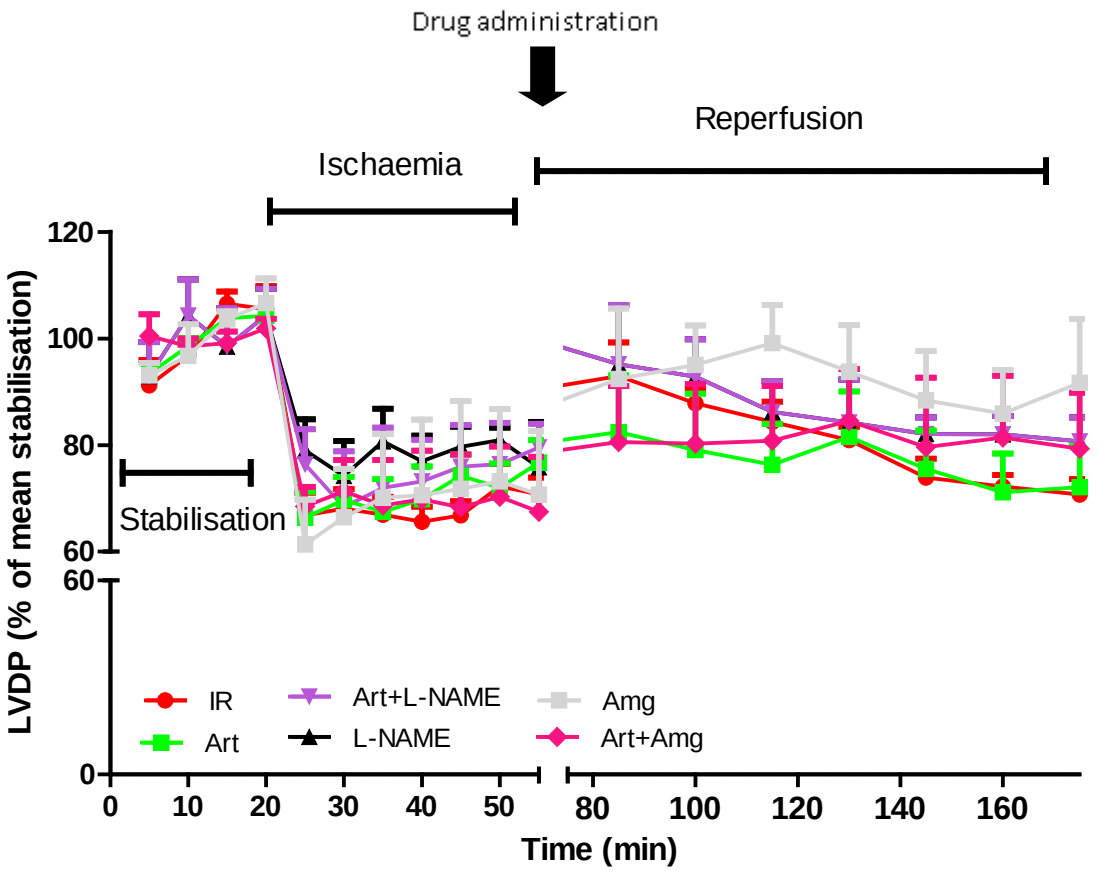


Figure 43. *The effects of artemisinin (4.3μM) in the presence and absence of inhibitor, L-NAME (100μM) and aminoguanidine (100μM) on LVDP expressed as a percentage of mean stabilisation. Hearts underwent 20 minutes of stabilisation, 35 minutes ischaemia and 120 minutes of reperfusion in the presence/absence of*

artemisinin (4.3µM) ± inhibitors which were administered throughout reperfusion. Results presented are Mean±SEM (n=3-8).

LVDP remained relatively stable throughout stabilisation and ischaemia with control showing a steady decline over time during reperfusion compared to the drug treatment which is however not significant. Drug treatments did not have a significant effect on LVDP when compared to control.

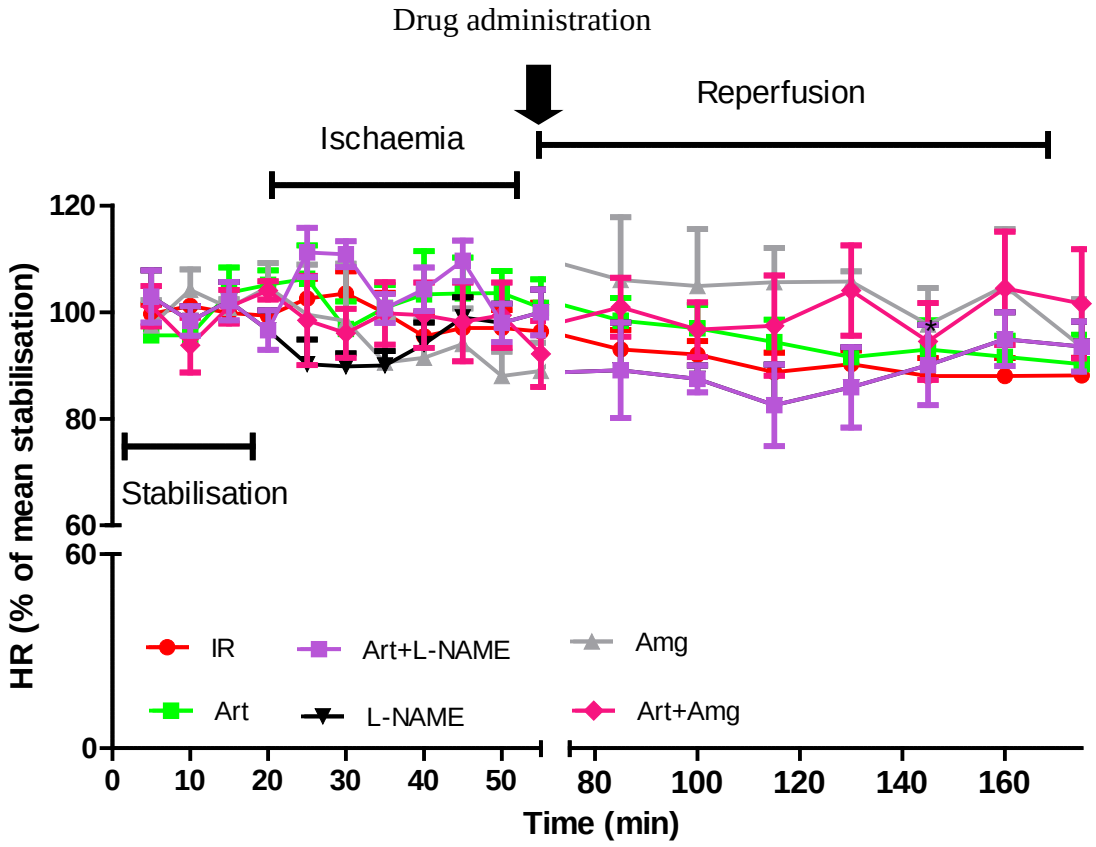


Figure 44. *The effects of Artemisinin (4.3µM), L-NAME (100µM) and aminoguanidine (100µM) on H/R (HR) as a percentage of mean stabilisation. Hearts underwent 20 minutes of stabilisation, 35 minutes of Ischaemia and 120 minutes of reperfusion in the presence/absence of Artemisinin (4.3µM) ± inhibitors. Art (4.3µM) was administered throughout the 120 minutes reperfusion with/without inhibitors (n=3-8).*

The H/R recorded was the number beats per minute (bpm) which showed no significant difference between the groups when compared with time matched controls.

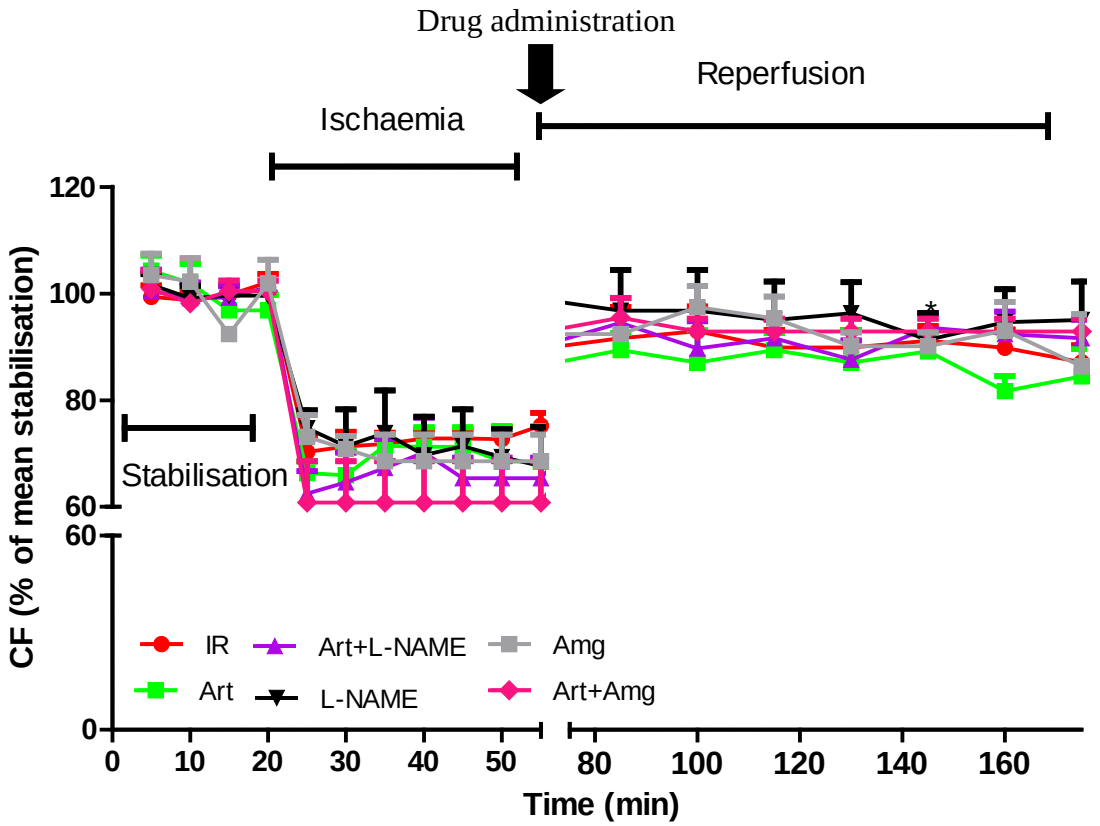


Figure 45. *The effects of artemisinin (4.3µM) in the presence and absence of inhibitor, L-NAME (100µM) and aminoguanidine (100µM) on CF (CF) expressed as a percentage of mean stabilisation .Hearts underwent 20 minutes of stabilisation, 35 minutes ischaemia and 120 minutes of reperfusion in the presence/absence of Artemisinin (4.3µM) ± inhibitors which were administered throughout reperfusion. Results presented are Mean±SEM (n=3-8).*

CF which was the amount of effluent (in mls) collected for duration of 1 minute at regular intervals of 5 minutes throughout the experiment. The results obtained were calculated as a percentage of mean stabilisation. CF recorded for the different treatment groups remained fairly regular throughout the experiment.

5.3.3 Artemisinin confers protection from I/R via activation of the Nitric Oxide cell survival pathway in the isolated perfused heart model

Administration of artemisinin (4.3µM) throughout reperfusion significantly ($P<0.001$) reduced infarct size by limiting myocardial ischaemia reperfusion injury (38.0±2.5% vs. 55.8±1.7%, Figure 46).

In order to evaluate the role of the nitric oxide pathway, a known cardioprotective pathway and also a downstream target of PI3K pathway. We administered artemisinin (4.3µM) with

non-selective nitric oxide inhibitor, L-NAME (100 µM), results showed a reversal in the artemisinin induced cardioprotection (44.7±0.9% vs. 38.0±2.5%, P<0.05 respectively, Figure 46). L-NAME alone (100µM) had no effect on infarct size to risk ratio compared to I/R control (52.0±1.5% vs. 55.8±1.7 %, P>0.05, respectively, Figure 46).

Co-administering artemisinin (4.3µM) with a selective iNOS inhibitor, aminoguanidine (100µM) throughout reperfusion significantly (P<0.05) reversed artemisinin's infarct sparing effects when compared to artemisinin (4.3µM) alone (48.3±1.4% vs. 38.0 ±2.5% respectively, Figure 46). Aminoguanidine alone (100µM) had no effect on I/R compared to I/R control (52.3±1.5% vs 55.8 ± 1.7% respectively, Figure 46).

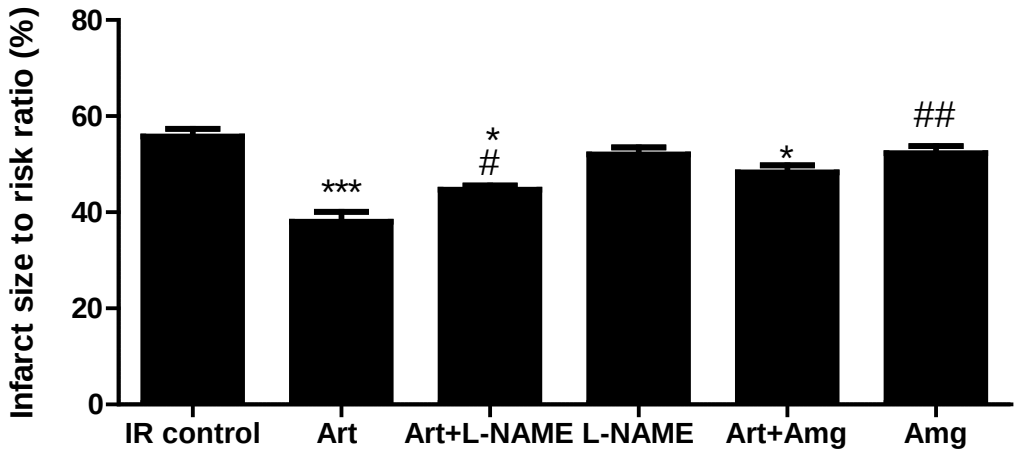


Figure 46*. Assessment of nitric oxide survival pathway in artemisinin (Art) mediated cardioprotection in isolated perfused heart model subjected to I/R. Art (4.3µM) was administered at reperfusion in the presence and absence of either L-NAME (non selective nitric oxide inhibitor)(100µM) or aminoguanidine (Amg) (iNOS inhibitor)(100µM). Results are shown as Mean±SEM.***P<0.001 vs. IR, *P<0.05 vs. Art, #P<0.05 vs. L-NAME, ##P<0.01 vs. Art. (n=3-8).*

5.3.4 Artemisinin improves the viability of isolated rat ventricular myocytes subjected to hypoxia/reoxygenation injury via nitric oxide signalling.

Cardiac myocytes were subjected to 2 hours of hypoxia and 2 hours of reoxygenation where artemisinin (4.3µM) was administered throughout the period of reoxygenation in the presence and absence of the non selective inhibitor of nitric oxide, L-NAME or selective iNOS inhibitor, aminoguanidine (Amg) (100µM respectively). Isolated ventricular mycoytes were also subjected to H/R and no treatment with a drug for control purposes.

Normoxic cells compared to H/R control showed a significant (P<0.001) decrease in cellular viability (100.0±0.0% vs 29.3±6.1%, respectively, Figure 47). However, artemisinin (4.3µM) administered throughout reoxygenation significantly improved cell viability compared to the H/R group (66.5±6.3% vs. 29.3±6.1 % respectively, P<0.01, Figure 47).

The non-selective inhibitor of nitric oxide synthase, L-NAME showed no significant effect on cellular viability when administered alone compared to H/R (47.0±7.1% vs. 29.3±6.1% respectively, Figure 47). However, by co-administering artemisinin (4.3µM) with L-NAME (100µM) the artemisinin induced cytoprotection was significantly (P<0.05) reversed (48.1 ±5.5% vs. 66.5 ± 6.3% respectively, Figure 47).

Aminoguanidine alone (100µM) however, did show significance when compared to H/R in terms of cellular viability (50.1±6.6% vs. 29.3±6.1%, P<0.05 respectively, Figure 47). The viability afforded to the cells by artemisinin similarly showed a significance (P<0.01) as previously described, in the presence of artemisinin (4.3µM) and aminoguanidine (100µM) however it was cellular viability was significantly decreased compared to artemisinin alone (46.4±6.2% vs. 66.5±6.3% respectively, P<0.05, Figure 47).

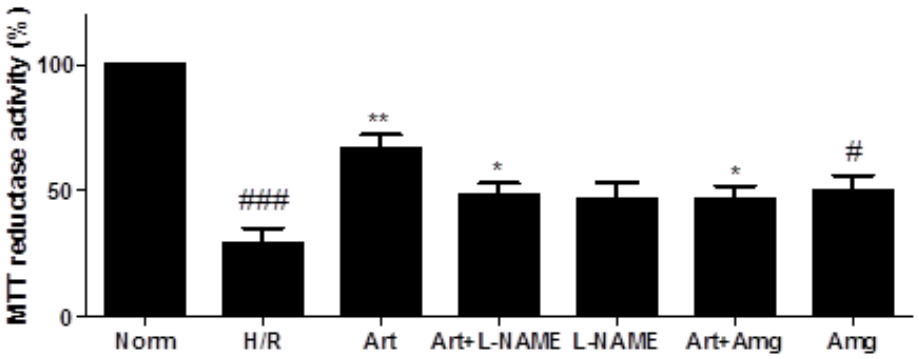


Figure 47. *MTT reductase activity used in assessing cellular viability in isolated ventricular cardiomyocytes subjected to 2 hours of hypoxia and 2 hours of reoxygenation. Where Art (4.3µM) was administered in the presence and absence of L-NAME (100µM) and aminoguaninde (100µM). ### P<0.001 vs Norm, **P<0.01 vs H/R, *P<0.05 vs Art ,#P<0.05 vs H/R (n=3-8).*

5.3.5 Artemisinin decreases cleaved caspase-3 activity in isolated ventricular cardiomyocytes subjected to H/R injury via the nitric oxide cellular survival pathway

In order to determine whether artemisinin at reperfusion can attenuate caspase 3 activity, in an adult rat cardiomyocyte model of H/R injury artemisinin (4.3µM) was administered throughout reoxygenation following hypoxia. The study also investigated the role of nitric oxide synthase, eNOS and iNOS by administering inhibitors of nitric oxide, L-NAME (100µM) and aminoguanidine (100µM) respectively alone and in combination with artemisinin (4.3µM) during reoxygenation to determine the involvement of the nitric oxide pathway in the mediated cytoprotection observed.

Isolated rat ventricular myocytes were subjected to 2 hours of hypoxia followed by 120 minutes of reoxygenation. Cells at reoxygenation were then treated with artemisinin (4.3µM) alone and in the presence/absence of L-NAME (100µM) or aminoguanidine (100µM) from which we assessed the levels of cleaved caspase-3 via flow cytometric analysis. The normoxic group had a low level of activated caspase-3 while the H/R group showed a significant ($P<0.001$) increase in the levels of cleaved caspase-3 activity compared to the normoxic control (8.8±1.2% vs. 26.8±2.0%, respectively, Figure 48). Treatment with artemisinin (4.3µM) showed a significant ($P<0.05$) decrease in the levels of caspase-3 compared to H/R control (17.1±2.0% vs. 26.8±2.0%, respectively, Figure 48). Co-treatment of artemisinin (4.3µM) with L-NAME significantly ($P<0.01$) reversed the anti-caspase 3 effect of artemisinin (31.0±1.4% vs. 17.1±2.0%, respectively, Figure 48). Co-administering artemisinin (4.3µM) with aminoguanidine (100µM) when compared to artemisinin showed a significant decrease also (26.7±3.2% vs. 17.1±2.0%, respectively, $P<0.05$, Figure 48). However, treatment with L-NAME showed, L-NAME had no significant effect compared to H/R control (29.9±0.7% vs. 26.8±2.0%, $P>0.05$, Figure 48). No significant effect was observed with aminoguanidine administered alone throughout reoxygenation too compared to H/R control (28.0±2.1 vs. 26.8±2.0%, $P>0.05$, Figure 48).

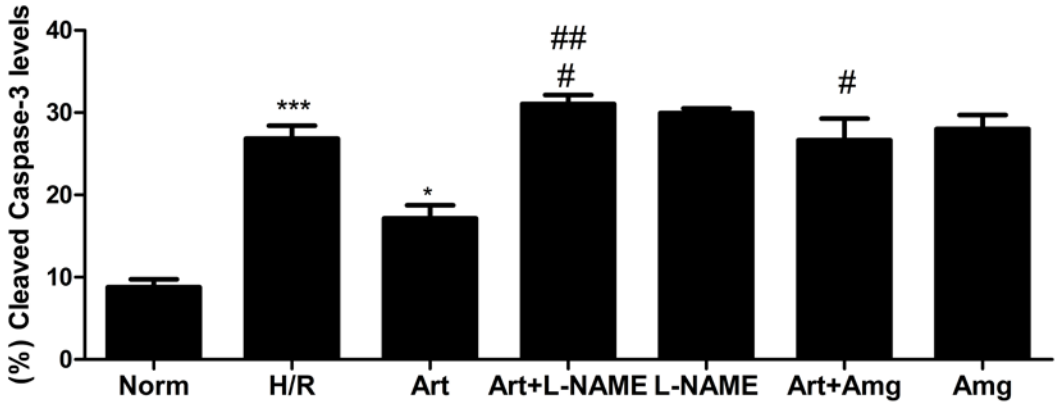


Figure 48*. Effect of administration of artemisinin (Art) (4.3µM) in the presence and absence of L-NAME (100µM) and aminoguanidine (Amg) (100µM) on cleaved caspase-3 levels as analysed by Flow Cytometry. ***P<0.001 vs Norm, *P<0.05 vs H/R, ##P<0.01 vs. Art, #P<0.05 vs. Art. (n=4-6)*

5.3.6 Artemisinin mediates cardioprotection via upregulation and activation of eNOS (Ser1177) observed isolated ventricular cardiomyocytes subjected H/R.

Following isolation, cardiomyocytes were subjected to 120 minutes hypoxia and 120 minutes reoxygenation with artemisinin (4.3µM) treatment to determine the levels of p-eNOS expression using FACS analysis. Untreated cardiomyocytes subjected to H/R showed a decrease in the expression of p-eNOS compared to the normalised normoxic control (46.3±2.0% vs. 100.0±0%, P<0.001, Figure 49). Upon treating the cardiomyocytes with artemisinin, a significant (P<0.001) increase in the level of p-eNOS compared H/R control was observed (156.3± 31.0% vs. 46.3±2.0% respectively, Figure 49). Co-treating cells with non selective nitric oxide inhibitor, L-NAME (100µM) with artemisinin (4.3µM) upon reoxygenation, significantly (P<0.01) downregulated eNOS expressed nitric oxide (59.6±8.1% vs. 156.3± 31.0%, Figure 49). Treatment with L-NAME (100µM) alone upon reoxygenation had no effect on p-eNOS expression compared to H/R control (51.0±10.6% vs. 46.3±2.0%, Figure 49).

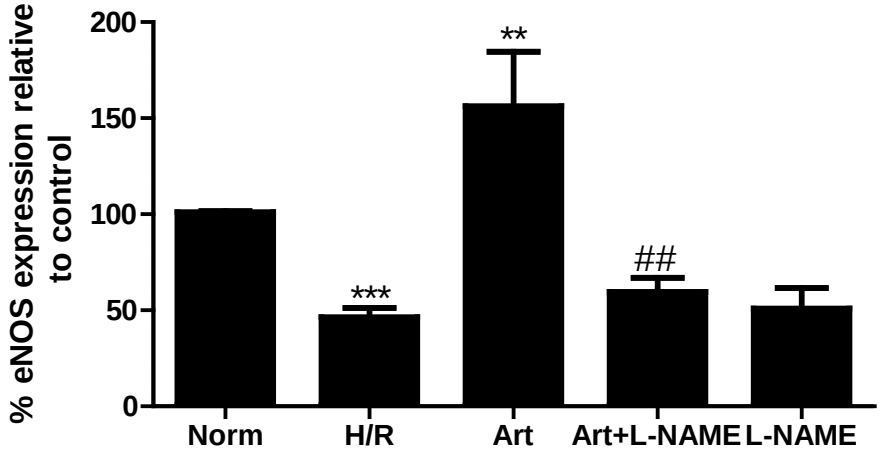


Figure 49. *Effect of administering artemisinin (Art) (4.3μM) throughout reoxygenation in the presence and absence of L-NAME (100μM) on $_{(Ser\ 1177)}$ expressions as analysed using Flow Cytometry. ***P<0.001 vs Normoxia (Norm), **P<0.01 vs H/R, ##P<0.01 vs. Art (n=4-6).*

5.3.7 Artemisinin treatment in isolated cardiomyocytes shows elevation in iNOS expressions.

Isolated ventricular cardiomyocytes subjected to after 2 hours hypoxia, 2 hours reoxygenation presented a reduced level of iNOS compared to normoxic control (69.9 ± 13.5 % vs. 100.0±0%, P<0.05, Figure 50). Whereas treatment with artemisinin (4.3μM) showed a significant (P<0.01) increase in the level of iNOS compared H/R control (150.4 ± 14.4% vs. 69.9 ± 13.5% respectively, Figure 50). Co-treating cells with L-NAME (100μM) and artemisinin (4.3μM) significantly (P<0.01) inhibited $eNOS_{(Ser\ 1177)}$ expression (71.5 ± 5.8 % vs. 150.4 ± 14.4%, Figure 50) however administering L-NAME alone had no effect on iNOS expression as compared to H/R control (72.8 ± 14.0 % vs. 69.9 ± 13.45 %, Figure 50)

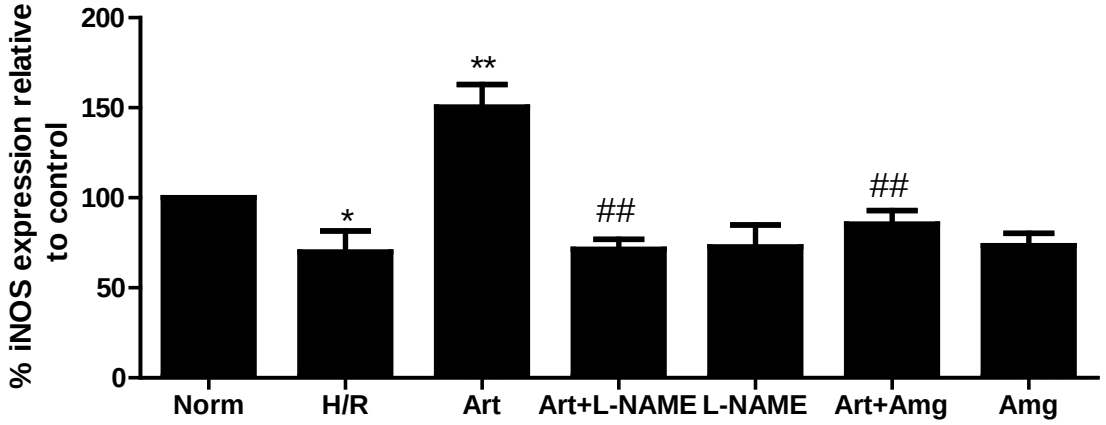
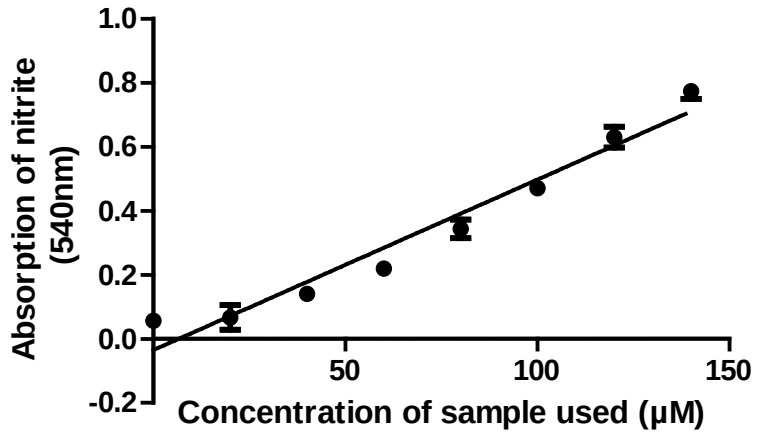


Figure 50: *Effect of artemisinin (Art) (4.3μM) on iNOS expression in the presence and absence of L-NAME (100μM) and Aminoguanidine (Amg) (100μM) analysed using FACS analysis. *P<0.05 vs. Normoxia (Norm), **P<vs. HR, ##P< vs. Art (n=4-6)*

5.3.8 Increase in nitrite and nitrate levels in artemisinin treated cardiomyocytes

The OxiSelect™ nitric oxide (Nitrite/Nitrate) assay is a simple, colorimetric assay that quantitatively measures nitric oxide in various samples by NO_2^-/NO_3^- (nitrite/nitrate) determination. Results show nitrate in the sample converted to nitrite by nitrate reductase enzyme which is detected with Griess Reagents as a colored azo dye product (absorbance 540nm).



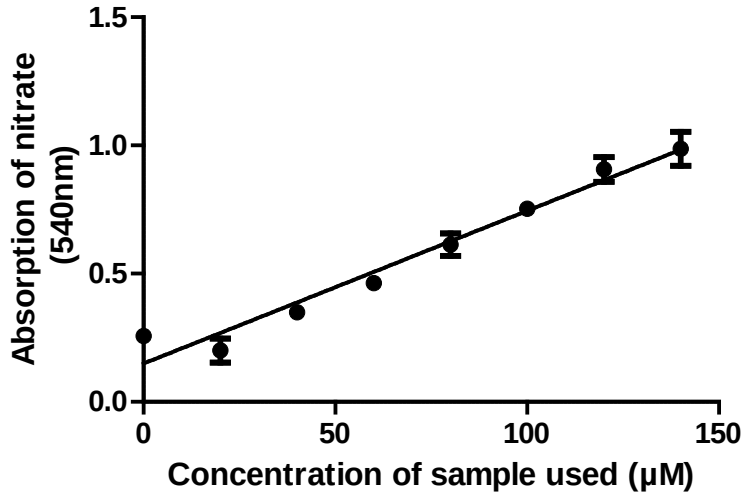


***Figure 51a and 51b**. Nitrite and Nitrate Standard Curves. Nitrite (left) and nitrate (right) standard curves were performed according to the OxiSelect™ in vitro nitric oxide colorimetric assay protocol showing a directly proportional increase in the absorbance and concentration of nitrite and nitrate in control samples respectively. Results expressed as Mean±SEM.*

Figure 51a and 51b show the absorbance of the nitrate and nitrite standard solutions with which the drug treated cardiomyocytes were measured. Table 4a shows the average of control and artemisinin treated cardiomyocytes (n=4-6). The concentration of nitrate within each sample was calculated by comparing the sample absorbance to the standard curve. Negative controls (without nitrate) were subtracted as shown in Table 4 thus showing the effect of artemisinin treatment in this assay. Data was collected for artemisinin treatment without inhibitors as the results were inconclusive and showed no obvious differences between treatments.

Concentration (µM)	**Control**	**Artemisinin**
0	0.19	0.20
20	0.06	0.22
40	0.17	0.34
60	0.25	0.36
80	0.32	0.34

100	0.29	0.43
120	0.29	0.46
140	0.18	0.51

Table 4*. Table showing the difference in mean absorbance of nitrate and nitrite in artemsinin treated cell lysate compared to standard control sample (n=4-6)*

5.4 DISCUSSION

In the previous chapter, it was confirmed that by administering artemisinin at reperfusion, artemisinin significantly alleviated evidence of myocardial injury and improved cellular survival in the myocardial model of I/R and H/R respectively. This findings are supported by research by Sun *et al*. (2007) who showed artemisinin possesses some cardioprotective effects by limiting infarct size development (Sun *et al*., 2007). More recently, many researchers have reported artemisinin's great potential against several cancer types (Yatuv *et al*., 2010; Lai and Singh 2006; Qadieri *et al*., 2013). However, this is under researched in terms of its effects in the heart, which is a popular organ target of toxicity in presently used anthracyclines (Toldo *et al.,* 2013). The main finding of this study is establishing the downstream targets of the pathway PI3K-Akt-nitric oxide cell survival pathway in artemisinins' mediated cardioprotection in cardiomyocytes and in HL-60 cells.

Activating Akt can influence a variety of downstream targets that can have multiple effects on cellular function especially on kinases such as p70S6K, eNOS, and other downstream targets (Kane and Weiss, 2003; Jonassen *et al.,* 2004). Akt is known to activate pro-survival factors as well as inhibit the activation of caspase 3, thus decreasing the number of cells committed to apoptosis (Sun *et al*., 2012; Hussain *et al.,* 2013)

By investigating myocardial injury, apoptosis and the cellular factors and signalling pathways that regulate apoptosis, this study may ultimately enable the development of ways of suppressing apoptotic cell death and understand in-depth the process of myocardial injury and how to certain therapies can alleviate it. I/R injury, myocardial infarction, heart

failure and drug induced cardiotoxicity are amongst the main causes of apoptosis and necrosis in cardiac myocytes (Zhou *et al*., 2014).

The major finding of this chapter is that artemisinin significantly alleviates myocardial injury of simulated myocardial I/R and H/R. Results from the isolated perfused hearts showed that artemisinin decreases infarct size, improves myocyte viability and decreases cleaved-caspase-3 activity in isolated ventricular myocytes. From the different models investigated in this study, it was established that the cardioprotective properties of artemisinin were as a result of activation of nitric oxide following the induced ischaemic/hypoxic injury.

Myocardial damage caused as a result of ischaemia and reperfusion therapy has been associated with free radical-induced myocardial damage, neutrophil accumulation, intracellular calcium overload, endothelial and microvascular dysfunction, and altered myocardial metabolism (Verma *et al.,* 2002; Monassier 2008). Although not investigated in our I/R and HR studies, it explains the mechanism behind the induced damaged. The injury induced is as a result of the metabolic and functional changes occurring in the myocardium (Rosano *et al.,* 2008). A decrease in ATP, creatine phosphate (CP) and changes in pH causes the proteins within cells to denature, subsequently resulting in an overall change in the working heart model (Kubes and Granger 1993; Wright *et al.,* 1995).

Studies have postulated that exposure to hypoxia favours an increase in the production of ROS in the mitochondria, activation of NADPH oxidase, xanthine oxidase/reductase, and nitric oxide synthase enzymes production as well as cellular hypertrophy (Araneda and Tuesta 2012). The production of nitric oxide during ischaemia has led to a lot of controversy in studying whether nitric oxide plays a protective or detrimental role in myocardial I/R injury (Ozaki *et al*., 2002; Kanno *et al*., 2000). Literature postulating that nitric oxide plays a ubiquitous role in modulating biological processes ranging from normal biological processes to pathophysiological extremes is extensive (Rakhit and Marber 2001). Injury induced by I/R directly damages myocytes; this is attributed to altered nitric oxide formation (Bredt and Snyder 1994). Nitric oxide is known to affect diverse physiological and pathological processes within the biological system (Sharma *et al*.,

2008). Recent findings do not support the controversy of the detrimental effects of nitric oxide. It is well established that nitric oxide plays an important role in maintaining cellular physiology and cytostasis (Ramirez *et al.*, 2010).

Furthermore, there is overwhelming evidence showing that nitric oxide is an important cardioprotective molecule via its vasodilator, antioxidant, antiplatelet, and anti-neutrophil actions making it essential for normal cellular homeostasis (Ferdinandy 2006). However, nitric oxide is only considered detrimental if when it combines with O_2 it forms $ONOO^-$ (Palcher *et al.*, 2007). This rapidly decomposes to generate highly reactive oxidative intermediates. iNOS has been shown to mediate anti-stunning and anti-infarct actions which have been elicited stimulus such as ischaemia (Bolli 2001).

In normal physiological conditions, there is a critical balance between cellular concentrations of nitric oxide, O_2, and superoxide dismutase, which favour nitric oxide production (Fulda *et al.*, 2010). In conditions such as I/R, the formation of ONOO- is favoured. However, in order to induce preconditioning, which involves brief episodes of ischaemia, both NO and O_2 synthesis are required (Ferdinandy and Schulz 2003). The overproduction of NO, $O_2´$ and ONOO during subsequent episode of ischaemia and reperfusion is attributed to the cardioprotection observed in preconditioning in which model (Ferdinandy and Schulz 2003).

In the different models evaluated by this study, L-NAME, a non selective inhibitor of nitric oxide synthase was administered alone and in combination with artemisinin. Several studies have shown L-NAME effectively blocks nitric oxide synthesis (Boultadakis and Pitsikas 2010; Minami *et al*., 1995; Klamer *et al*., 2001). In the isolated ventricular cardiomyocyte, L-NAME had no effect on infarct size whereas in combination with artemisinin, the cardioprotection observed with artemisinin were abolished. This present study was able to show artemisinin's capacity in attenuating myocardial ischaemia/reperfusion injury while implicating the nitric oxide pathway. Studies have supported our findings by showing the administration of L-NAME in addition to flavonoids partially abolished the cardioprotection afforded by the flavonoids against I/R in rats, which suggests the role of nitric oxide in cardioprotection (Challa *et al*., 2010). This, supports that

an upregulatory effect on eNOS leading to increased nitric oxide production is cardioprotective as observed with artemisinin treatment. Evidence from Felaco *et al*. (2000) also supports that an increase in the levels of p-eNOS contributes to the mechanism of cardioprotection against I/R injury as we have also observed with artemisinin-treatment in cardiomyocytes.

Furthermore, study by Bell and Yellon (2002) showed that by administering bradykinin in an isolated perfused rat heart model, the PI3K-Akt pathway is activated resulting in an increase in eNOS nitric oxide leading to an ultimately a decrease in infarct size and cardioprotection in the treated hearts.

Gao *et al*. (2002) supported our findings by showing that administering insulin *in vivo* reduced myocardial apoptosis via the activation of p-eNOS and increased nitric oxide production through the PI3K-Akt pathway (Gao *et al*., 2002). Additionally, further studies by Gao's group (2005) have shown that treatment with glucose-insulin-potassium (GIK) at reperfusion recruited the same Akt-dependent and NO-mediated pathway of protection (Ma *et al*., 2006). Recent studies by Smart *et al*. involving pre-treatment of cardiomyocytes with interleukin-6 (IL-6) have similarly induced a PI3K and NO-dependent pathway of protection with an increase in expression of iNOS (Smart *et al*., 2006).

We went on further to analyse the effects of I/R and H/R on iNOS expression in isolated perfused hearts and ventricular rat cardiomyocytes, respectively. Artemisinin (4.3μM) and L-NAME (100μM) were administered together during reperfusion/reoxygenation. Similarly, we were able to attribute the nitric oxide dependent protection to iNOS isoform expression, whereby artemisinin administered alone reduced infarct size/cellular viability following I/R or H/R injury and more importantly the observed protection was abrogated by aminoguanidine, a highly specific iNOS inhibitor. Despite the increase in eNOS/iNOS expression with drug treatment, aminoguanidine/L-NAME did not significanty decrease eNOS/iNOS levels compared to H/R control. Our findings are supported by Li *et al*. (2003) who demonstrated iNOS overexpression (through adenovirally encoded human iNOS) to be greatly protective against I/R injury in mice (Li *et al*., 2003). Heger *et al*. (2002) have also analysed mice with constitutively expressed cardiac-specific overexpression of iNOS

and found that the overexpression of iNOS conferred protection following ischaemia-reperfusion in isolated hearts (Heger *et al*., 2002). Furthermore, Zingarelli *et al*. confirmed that the absence of the iNOS gene leads to the development of tissue damage in ischemic hearts after early reperfusion (Zingarelli *et al*., 2002) thus concluding that iNOS plays a beneficial role in modulating the early defensive inflammatory response against reperfusion injury through regulation of signal transduction. These finding offers an explanation to the artemisinin-mediated protection observed in the I/R model which we associated with the nitric oxide-dependent pathway, in particular the upregulation of iNOS.

Also in the cleaved caspase-3 assay, isolated ventricular rat myocytes were stained using an intracellular Alexa Fluor® 488 labelled caspase-3 antibody which detects myocyte cleaved caspase 3 activity. Our results demonstrated that H/R caused an upregulation of activated caspase-3 levels when compared to normoxic controls whereby treatment with artemisinin caused a decrease in caspase-3 activity. During the activation of the caspases, firstly, an initiator caspase is activated as a result of hypoxia is then cleaved and in turn activates the effector caspases leading to a proteolytic cascade that initiates cell death as picked up in this assay. The levels of cleaved caspase-3 activity were measured via flow cytometry in this study indicate cells potentially committed to apoptosis (Jovanovic *et al*., 2002). Feng et al. (2005) using bradykinin similary showed that preconditioning the heart with bradykinin would improve survival via upregulation in antiapoptotic proteins and inhibition of myocardial apoptosis in rabbit hearts subjected to 30 minutes simulated heart arrest using cardiopegia ischaemia. This study relates apoptosis to cleaved caspase-3 activity and showed the decrease in the levels of activated caspase-3 shows a decrease in the number of cells committed to apoptosis while associating it to protection.

Due to its ubiquitous role, nitric oxide has also been associated with inducing apoptosis in cardiac myocytes in a dose-dependent manner. In high concentrations, nitric oxide can induce caspase activation, DNA fragmentation and cell death (Choi *et al*., 2010; Jovanovic *et al*., 2002). Our results also suggests that upregulation of nitric oxide, induced by administering artemisinin at reperfusion, is involved in inhibiting caspase-3. This is evident in the use of inihibitors of nitric oxide such as L-NAME and aminoguanidine. It is well documented in human endothelial cells that nitric oxide completely abrogates apoptosis by

interfering with the activation of the caspase cascade (Dimmeler *et al*., 1997). In *in vivo* studies, nitric oxide was shown to inhibit caspase-3 through S-nitrosation (Rossig *et al*., 1999).

Mitochondrial studies have also suggested that mitochondrial damage may be potentiated by nitric oxide, which leads to activation of the apoptotic-signalling cascade. Whilst mitochondria appear to be a target for nitric oxide-mediated injury, the mitochondria may also be a potential target for protection (Borutaite *et al*., 2000). Due to the vast advances in understanding the role of nitric oxide in the ischaemic biology of the heart, it is well established that nitric oxide prevents I/R, although the mechanism is debatable (Ferdinandy *et al.*, 2003). However, studies have suggested that nitric oxide functions via the inhibition of the respiratory chain depolarization of the mitochondrial membrane or from the prevention of ROS generation and calcium uptake in cells (Gao *et al*., 2008; Camara *et al*., 2010) which explains the protective properties it displays. Which we postulate may equally explain artemisinin's mediated protection we have observed.

Ferdinandy *et al*. (2007) determined that increase in ROS may significantly disrupt major cytoprotective signalling pathways (Ferdinandy *et al*., 2007) with nitric oxide being a critical mediator of myocardial homeostasis such as vasodilation and adaption to stress (Jones and Bolli 2006). Nitric oxide is sufficient to confer cardioprotection as research has established, with growing studies suggesting that nitric oxide bioavailability is necessary in eliciting several protective interventions (Jones *et al*., 2003). In this chapter, we have successfully shown an increase in nitric oxide with artemisinin treatment in the different assays employed. These findings are similar to a study by Salloum *et al*. (2003) which showed that sildenafil increased eNOS and iNOS proteins in the heart (Salloum *et al*., 2003) and cardiomyocytes via the opening of the mitochondria opening of mitochondrial ATP-dependent K^+ channels. Das *et al*., (2005) have also shown that sildenafil protects against necrosis and apoptosis through Nitric oxide signalling pathway which has a possible therapeutic potential in preventing myocyte cell death following I/R. This has a direct effect on protection against myocardial infarction (Salloum *et al*., 2003) and simulated I/R injury and apoptosis in cardiomyocytes (Das *et al*., 2005; Kukreja 2007). Evidence from cultured endothelial cell obtained from male Sprague Dawley rat subjected

Insulin treatment following I/R has also affirmed that phosphorylation of eNOS via PI3K/Akt pathway increases nitric oxide production (Gao *et al*., 2002). This therefore elucidates the importance of nitric oxide signalling pathway and its anti-apoptotic effect following myocardial ischaemia and reperfusion.

eNOS is essential in determining the vascular tone and platelet activity in cells. However, it's most important in the modulation of myocardial metabolism by nitric oxide (Trochu *et al*., 2000). Our results show artemisinin stimulates p-eNOS, supported by Bell and Yellon (2003) who have shown by using bradykinins in an isolated perfused rat model of ischaemia reperfusion that the recruitment of PI3K and Akt leads to the phosphorylation of eNOS which attenuates reperfusion injury (Bell and Yellon 2003). The presence of nitric oxide in our artemisinin treated cells has been previously attributed to cardioprotective properties in different models. Evidence from research has also shown a decrease in the amount of basal nitric oxide following inducing ischaemia (Maulik *et al*., 1995) this suggests the increase amounts seen in comparison to the I/R control group was as a result of the drug treatment. Bell and Yellon 2003 have shown that the presence of eNOS is a prerequisite to the drug-mediated salvaging effect in reperfusion injury implying a role of nitric oxide in the observed protection. Additionally, Liu *et al*. (2002) have emphasised and highlighted the important role of eNOS in limiting post infarction cardiac hypertrophy. Other studies have also confirmed exposure to hypoxia causes a reduction in eNOS levels due to the generation of reactive oxygen species, lipid peroxidation, protein denaturation and DNA damage (Cacciutolo *et al*., 1993). Likewise, increased phosphorylation of eNOS in our artemisinin treatment groups significantly improved/restored myocardial function, which is in line with previous studies on eNOS upregulation (Kukreja 2007; Farah *et al*., 2013). This may be due to the blocking of nitric oxide synthase and superoxide formation caused by eNOS, (Pou *et al.*, 1992) hence resulting in cardioprotective effect.

Other analytical techniques have also been used to quantitate the major metabolites of nitric oxide i.e. nitrite and nitrate, in a variety of biological fluids (Tsikas 2007). To quantitatively measure nitrite and nitrate levels (which are the oxidation products of nitric oxide), Griess reagent has been exclusively used for decades now to measure nitrite levels. To quantify

nitrate, nitrate is enzymatic reduced to nitrite which is inidicative of nitric oxide production (Chartterjee *et al.,* 2002).

Our results show artemisinin treatment led to an increase in the levels of nitrate and nitrite in a dose-dependent manner. Laboratory studies mimicking myocardial infarction have shown an increased induction of iNOS, eNOS, and nitric oxide in the heart, followed by increased plasma concentrations of nitrate and nitrite (Feng *et al*., 2001). Nevertheless such studies emphasised that the balance between the cytostasis and cytotoxicity of nitric oxide is dependent on the amount of nitric oxide synthase available within the tissue as well as the interactions with free radicals such as the superoxide (Rakhit and Marber 2001). Interestingly, our results showed an increase in iNOS with artemisinin treatment. Upregulation of nitric oxide by iNOS however has been previously linked to myocardial dysfunction and results in higher mortality after myocardial infarction (Feng *et al.,* 2001). Contrastingly, Aldieri *et al*. (2003), showed that iNOS upregulation in cells of neonatal rats was not followed by any associated toxicity as it is usually following the reaction with superoxide to form peroxynitrate which supports the findings in rat cardiomyocytes. By altering mitochondrial permeability, there is an increase in the pro apoptotic Bcl-2 family (e.g. Bax), which then stimulates apoptosis (Haunstetter and Izumo 1998; Mashimo and Goyal 1999).

However, studies by other researchers such as Xiong *et al*. (2010) have supported our findings and shown that artemisinin can ameliorate cardiac hypertrophy after aortic banding. Due to artemisinin's low toxicity profile, it is a favourable drug of choice in anti-malarial therapy and with great potential in a variety of settings. Furthermore, studies have shown artemisinin and its derivatives are increasingly being used due to their reliable and consistent pharmacokinetic profiles (Pasvol 2005).

5.5 CONCLUSION

Developing pharmacological approaches to attenuate the incidence of I/R and delaying the onset of apoptosis and necrosis is of great clinical importance (Ferdinandy *et al*., 2007). We can therefore conclude the pharmacological agents such as artemisinin, widely used in

the clinical setting as an anti-malarial agent; in the myocardium has the ability to reduce infarct size and ischaemia-reperfusion injury in a nitric oxide-dependent manner.

Chapter 6

6 ARTEMISININ PROTECTS AGAINST DOXORUBICIN-INDUCED CARDIOTOXICITY AND ENHANCES CYTOTOXICITY IN HL-60 CANCER CELLS VIA THE NITRIC OXIDE SIGNALLING PATHWAYS

6.1 INTRODUCTION

The use of anthracyclines alone or in combination with other anti-cancer treatments remains the most viable chemotherapy treatment for a range of cancers, including breast cancer, leukaemia, lymphomas and sarcomas (Vejpongsa and Yeh, 2013). Cancer chemotherapy has made remarkable advances over the years in the treatment of solid and haematologic malignancies such as using immunoconjugates (which are cytotoxic effectors covalently linked to monoclonal antibodies that enable a more targeted cell-surface receptor recognition delivery system) (Wessels and OW, 2014). Encouraging results have also been yielded with the use of immunotherapies particularly immune checkpoint inhibitors in several cancer types, however immunotherapies are not here to replace chemotherapy or use of anthracyclines which has undoubtedly revolutionised cancer therapy (Volkova and Russell, 2011; Armand 2015). Anthracyclines have emerged as one of the most effective anticancer treatments ever developed. Unfortunately, their clinical use is limited due to their associated cardiotoxicity (van Dalen *et al.*, 2008).

Doxorubicin, a widely used anthracycline, is used in treatment against leukaemia, lymphoma, and breast and prostate cancer (Liu *et al.*, 2008; Torres and Simic 2012). Doxorubicin has been shown to have a cumulative dose-dependent cardiotoxicity, with the risk of heart failure increasing significantly after the cumulative dose increases above

400 mg/m^2 (Vejpongsa and Yeh, 2013). Doxorubicin therapy is therefore limited due to acute or chronic toxicity to the heart, with the acute toxicity being transient and clinically manageable while the chronic toxicity evolves into progressive cardiomyopathy, thus limiting its clinical use (Minotti *et al.*, 2001). Studies have shown that treatment with doxorubicin often results in increased risks of cardiomyopathies, congestive heart failure and subsequently an increased risk of mortality in cancer patients (Koti *et al.*, 2008; Vergely *et al.*, 2007). This has led to several deaths as a result of non cancer causes especially in patients with pre-existing cardiac conditions such as ventricular dysfunction (Takemura and Fujiwara 2007; Adams and Lipshultz 2005; Tacar et al. 2013). Other risk factors that may increase the risk of anthracycline induced cardiotoxicity that have been identified as being age, female gender, prior mediastinal radiation therapy, hypertension, myopathy and or heart failure (Hoff et al., 1979; Volkova and Rusell 2011).

Cardiac toxicity is one of most detrimental side effects of anticancer therapies, so the gain in life expectancy due to anticancer therapy might be countered by increased mortality due to cardiac problems such as heart failure, myocardial ischaemia, arrhythmias, hypertension or thromboembolism (Metens *et al.*, 2008, Bovelli *et al.*, 2010). Several studies have confirmed that cancer patients treated with doxorubicin develop adverse cardiac side effects that often led to the termination of doxorubicin treatment (Kalyanaraman *et al*., 2002; Singal *et al.*, 2001). Statistical evidence gathered by oncologists assessing the risk of cancer relapse suggest using adjuvant therapy such as chemotherapy, radiation therapy, hormone therapy or an adjuvant anthracycline in addition to primary treatment maximises efficiency of treatment thus improving survival and limiting specific side effects (Handa 2014; Peters 1993; Bonadona and Valagussa 1981). Although, limited data is available of the reoccurrence of cancers and of the benefits of adjuvant treatments (Sargent *et al*., 2008).
Adjuvant chemotherapy has been shown to provide several benefits for example Trastuzumab is a monoclonal antibody that targets the human epidermal growth factor receptor 2 (HER2) protein (Slamon *et al.*, 2001). Trastuzumab is approved for patients with HER2-positive breast cancer and is given for treatment of metastatic cancers and very often as an adjunctive therapy for breast cancer treatment (Romond *et al.*, 2005). Initial studies on trastuzumab showed that it improved survival in the late-stage of HER2-positive breast cancer (Hudis 2007). If treatment is started at the early stages trastuzumab also reduces the

risk of cancer recurring after remission from 9.5% to 3% however studies have also shown that it increases the risk of cardiovascular events (Moja *et al.,* 2012). Another clinical trial also showed that administering doxorubicin and cyclophosphamide followed by adjuvant trastuzumab resulted in an increased incidence of congestive heart failure due to the adjunctive treatment (Russell *et al.,* 2010). This further emphasises the urgent need to develop a safe and effective treatment against the different malignancies without compromising on the antineoplastic efficacy of doxorubicin (Scott *et al*., 2011).

The mechanism of doxorubicin induced cardiotoxicity appears to be multifactorial and has been shown to involve a variety of processes such as oxidative stress, nitrosative stress, and ROS. In addition to ROS formation, RNS are also implicated in doxorubicin cardiotoxicity (Mukhopadhyay *et al.,* 2007). The stress placed on the cardiomyocytes by the free radicals involves doxorubicin and its metabolites such as doxorubicinol which has been implicated in cardiotoxicity (Romana *et al*., 2008, Giorgio *et al*., 2004). Treatment with doxorubicin has also been previously shown to form complexes with iron which forms radicals (Stefano *et al*., 2004).

Research has suggested the cardiotoxicity is also linked to disruption of cardiomyocyte integrity and loss of cardiac function. Nitric oxide, an important radical gas is involved in cellular signalling, normal homeostasis as well as pathophysiology of heart (Hummel *et al*., 2006).

Previous chapters have associated artemisinin with nitric oxide and mentioned the potential of artemisinin to be effective against a variety of cancer types. It remains an imperative need to develop anti-cancer agents that are free from adverse cardiac side effects or the development of adjunctive therapies that protect the heart from cardiotoxicity. We therefore focused this chapter on the effects of artemisinin on HL-60 cells and in the myocardium as well as artemisinin's effect when co-administered with the severely cardiotoxic doxorubicin.

Studies have previously implicated nitric oxide in anthracycline toxicity, with nitric oxide playing a key role in the pathogenesis of diseases (Fogli *et al.,* 2014). Conversely, the beneficial nature of nitric oxide is well documented and has also been shown in a previous chapter (chapter 5) where nitric oxide was implicated in artemisinin mediated

cardioprotection. This conforms to the understanding that nitric oxide is a ubiquitous signalling molecule involved in a variety of physiological, biological and molecule events within cells (Hummel *et al*., 2006, Jones and Bolli 2006).

Due to the claims made by previous researchers in terms of the implication of doxorubicin and nitric oxide compounded with our earlier observed association of artemisinin mediated nitric oxide protection, we investigated the possible involvement of nitric oxide in doxorubicin's toxicity as well as that of artemisinin when used as an adjunctive therapy to doxorubicin in the myocardium and in HL-60 cell lines. We proposed that, artemisinin a known cardioprotective compound could potentially reverse doxorubicin induced toxicity by recruiting targets of the nitric oxide pathway without negatively impacting on its cytotoxicity in HL-60 cancer cells.

In this chapter, we therefore administered artemisinin at as an adjunctive therapy in HL-60 cells. This is with the aim of improving the doxorubicin associated toxicity due to artemisinins cardioprotective effect and its potential against several cancer types (Ho *et al*., 2012; Qaderi *et al.*, 2013). Artemisinin has been shown to be effective against 66 cancer cell types without the risk of exacerbating any cardiovascular conditions (Sun *et al.*, 2007; Kinoshita *et al*., 2010). We administered artemisinin in combination with doxorubicin and in the presence and absence of nitric oxide inhibitors (L-NAME and aminoguanidine) to conduct investigations in the different myocardial models previously tested as well as in HL-60 cells.

Aims of the current study were to investigate:

(a) the effect of artemisinin and doxorubicin administered during reperfusion in isolated perfused rat hearts and isolated ventricular cardiomyocytes subjected to I/R or H/R respectively. The study also investigated cell signalling pathway associated with the observed effect in the myocardium;
(b) the effect of co-administering artemisinin with doxorubicin in HL-60 cells while investigating the cell signalling pathway associated with the observed effect in the HL-60 cells;

(c) the effect of the adjunctive treatment on nitrite/nitrate levels in isolated cardiomyocytes.

6.2 METHODS

6.2.1 Animals

Adult male Sprague Dawley rats (350-400g) were obtained from Charles River (Margate, UK). The care and use of animals were in accordance with the Guidance on the Operation of the Animals (Scientific Procedures Act 1986). The study was carried out upon obtaining ethical approval from Coventry University Research ethics committee which was regularly assessed throughout the project.

6.2.2 Preparation of drugs

Artemisinin, L-NAME and aminoguanidine hydrochloride both supplied from Sigma-Aldrich (Poole, UK) and dissolved in DMSO making sure the final concentration of DMSO was less than 0.02% (a concentration shown not to affect haemodynamics or infarct size) (unpublished paper) and stored at -20 °C. Doxorubicin Hydrochloride purchased from Tocris Cookson (Bristol, UK) and was dissolved in ultrapure water aliquoted and stored at -20. MTT was purchased from Sigma (Poole, UK). Cleaved Caspase-3 (Asp175) Antibody (Alexa Fluor® 488 Conjugate), iNOS, phospho-eNOS $_{(Ser\ 1177),}$ total-eNOS and HRP conjugated Rabbit monoclonal antibodies and antibiotin were purchased from New England Biolabs (Hertfordshire, UK). SuperSignal West Femto® enhanced chemoluminescent substrates were purchased from Pierce (UK). The nitric oxide Assay was purchased from Cell Biolabs Inc (San Diego, USA). HL-60 cells were obtained from ECACC.

6.2.3 Isolated perfused rat heart model

Briefly, following sacrifice, the hearts were rapidly excised and cannulated as described in detai in chapter 2. Subsequent retrograde perfusion with KH buffer, saturated with 95% O_2, 5% CO_2, maintained at a temperature of 37 ± 0.5^{o}C and pH 7.4 using a water-jacketed heat exchange coil.

Balloon of a diastolic pressure of 8-10mmHg was inserted into the left atrium allowing LVDP to be measured. A physiological pressure transducer was connected to a bridge amp and a power lab (AD Instruments Ltd, Chalgrove, UK) which allows the LVDP, HR using ECG leads. CF was measured by collecting the perfusate at regular intervals.

The experiment was conducted for 175 minutes in total. 20 minutes stabilisation followed by 35 minutes ischaemia and 120 minutes reperfusion as described in chapter 2 (Figure 52).

Doxorubicin (1µM) and/or artemisinin (4.3µM) was administered throughout reperfusion in the presence and absence of either L-NAME (100µM) or aminoguanidine. TTC solution in 8ml of phosphate buffer 2 (100mM NaH_2PO_4) and 2ml phosphate buffer 1 (100mM $NaH_2PO_4.2H_2O$ for 10–12 minutes and fixed in 10% formaldehyde for at least 4 hours to enhance the staining prior to analysis.

The heart slices were then removed from formaldehyde and traced onto acetate film using different coloured markers to differentiate between the viable, at risk and infarct tissue which was scanned into a computer to allow calculation of Infarct to risk ratio from the differentiated tissues traced. Areas of viable, at risk and infarct tissue were measured using the Image Tool program as developed by UTHSCSA, Version 8.1.

6.2.4 Adult Rat Ventricular Myocytes Isolation

Adult ventricular rat myocytes were isolated from Sprague Dawley rats (350-400g) by enzymatic dissociation method (Maddock *et al*., 2002). Method described in detail in chapter 2. Hearts were mounted onto a modified Langendorff apparatus and perfused with modified Krebs buffer containing (in mM) ; 116 NaCl, 5.4 KCl, 0.4 $MgSO_4.7H_20$, 10 glucose, 20 taurine, 5 pyruvate, 0.9 Na_2HPO_4. $12H_2O$ and 25 $NaHCO_3$ 25 dissolved in RO water. The buffer was then oxygenated with 95% O_2 and 5% CO_2 and maintained at 37^oC, pH 7.4 using NaOH. The hearts were then perfused with a collagenase digestion buffer (containing Collagenase 0.075% (Worthingtons Type II) and 4.4M $CaCl_2$, pH 7.4) at a rate of 7.5ml/minutes. During perfusion with collagenase the effluent was collected and re-used.

Following digestion, the ventricles were mechanically disrupted and incubated for 10 minutes in 15ml of digestion buffer in an orbital shaker and oxygenated with 95% O_2 and

5% CO_2. Cells were then centrifuged at 400rpm for 2 minutes, supernatant discarded and the pelleted cells re-suspended in RB (in mM 116 NaCl, 5.4 KCL , 0.4 $MgSO_4$, 10 glucose, 20 taurine, 5 Pyruvate, 0.9 $NaHPO_4$, 5 Creatine, 2% BSA, 50µM $CaCl_2$ and 1% PenStrep pH 7.4 at 37^o C) where the calcium concentration was gradually brought to 1.25mM to avoid calcium overload. The isolated myocytes were incubated in RB (at 37^{o}C, 5% CO_2 for 24 hours before being used (Maddock *et al*., 2002)**.**

The cells were incubated in 15 mls of hypoxic buffer (12 mM KCL, 0.49 mM $MgCl_2$, 0.9 mM $CaCl_2$, 4 mM HEPES, 10 mM Deoxyglucose and 20 mM lactate and placed into a hypoxic chamber , Galaxy 48R (New Brunswick) pre-heated at 37 °C with 95% O_2 and 5% CO_2.. Myocytes were then assigned to the different treatment groups and were incubates with either: doxorubicin (1µM), artemisinin (4.3µM), doxorubicin (1µM) ± artemisinin (4.3µM), doxorubicin (1µM) ± artemisinin (4.3µM) ± L-NAME (100µM), doxorubicin (1µM) ± artemisinin (4.3µM) ± aminoguanidine (100µM) throughout the reoxygenation period. The myocytes then underwent reoxygenation for 2 hours. Upon completing reoxygenation the cells were then assessed either for cellular viability using MTT or underwent flow cytometric analysis for iNOS, p-eNOS (Ser 1177), total-eNOS or cleaved caspase-3 activity as described below.

6.2.5 Cellular viability assay based on MTT reductase activity using isolated ventricular cardiomyocytes

The cells were subjected to MTT assay which measures viable cells by reducing MTT tetrazolium dye from yellow to purple. The isolated myocytes were counted using a using a nucleo counter (Chemometec, Sartorius, Surrey, UK) and resuspended in restoration buffer (RB) to a density of 100,000 cells/ml. 1ml of the cells was pipetted to be used as normoxic control while the remaining cells were centrifuged and the pellet re-suspended in Esumi hypoxic buffer (in mM 137 NaCl, 12 KCL, 0.49 $MgCl_2$, 0.9 $CaCl_2$, 4 HEPES, 20 Na lactate, 10 deoxy-D-glucose). The myocytes were then incubated in a hypoxic chamber, Galaxy 48R (New Brunswick) for 120 minutes with atmosphere 5% CO_2 95% N_2 at 37^{o}C.

Some wells were used as normoxic control which contained 100µl of restoration buffer only. The remaining wells were randomly allocated to the different treatment groups which

contained 50µl of cells and 50µl of drug treatment. Drugs used for this study were diluted with restoration buffer to a final concentration of 4.3µM in the artemisinin treated group, 100µM in L-NAME treated group and 100µM in aminoguanidine treated group. The drugs were administered at the start of reoxygenation and cells incubated for an additional 120 minutes with 20µl of MTT (MTT solution consisting of 5mg.ml^{-1} in PBS (10g for 10^{-4} cells/well) was added at reoxygenation except for the blanks which contained restoration buffer and MTT solution only which were then incubated in the dark at 37^{0}C for 120 minutes. Myocytes were subsequently lysed with 100µl of lysis buffer (20% SDS in 50% dimethylformamide) and incubated on an orbital shaker and incubated overnight at 37^{o} C. Colorimetric analysis of the plate was done to measure the fluorescence emission at 450nm (Thermo Scientific, UK) using a plate reader (Anthos 2001). The absorbance was measured for the different treatment groups. The effect of the artemisinin treatment was obtained by subtracting the absorbance from the control values. Graphs were made using the mean absorbance of the drug treated group as a percentage of the mean absorbance of the control group.

6.2.6 Quantitative analysis for cleaved caspase-3, iNOS and eNOS (Ser 1177) using FACS analysis

Following the cardiomyocyte isolation, the myocytes were harvested and centrifuged at 1200 rpm for 2 minutes. The pellet was then resuspended in PBS and fixed with 3% formaldehyde for 10 minutes at room temperature. The cells were then put on ice for 1 minute before centrifuging at 1200 rpm for 2 minutes following aspiration of the supernatant. 250 µl of ice cold methanol (90%) was added and the samples were incubated on ice for 30 minutes before being washed twice in incubation buffer (0.5% BSA in PBS) following a 10 minutes (at 37°C) incubation of the samples each time followed by centrifugation of the samples (at 1200rpm, 2 minutes). The antibody was prepared to 1:100 final dilution in incubation buffer for the analysis of cleaved caspase-3 activity or iNOS and eNOS.

6.2.7 Quantitative analysis of cleaved caspase-3 activity

Cleaved caspase-3 (Asp175) antibody (Alexa Fluor 488 conjugate) was used to detect the levels of activated caspase-3 in the different experimental groups. For the analysis of cleaved

caspase-3 activity, the cells were incubated for 1 hour in cleaved caspase-$3_{(Asp175)}$ secondary rabbit monoclonal antibody (Alexa Fluor ® 488 conjugate) (New England Biolabs, Hertfordshire,UK) diluted at 1:1000. At the end of the incubation period, the cells were centrifuged and the supernatant was removed the cells resuspended in 500µl PBS and analysed using flow cytometer (Becton Dickinson, Oxford, UK) using the on the FL1 channel to count 10,000 events (Vermes *et al*., 2002; Hussain *et al*., 2014).

6.2.8 Quantitative analysis of iNOS, p-eNOS (Ser 1177) and eNOS

To quantify iNOS levels, FACS analysis was used to assess the differential protein levels for iNOS and GAPDH. Following centrifugation, the harvested cells samples were probed for 1 hour with iNOS or GAPDH rabbit monoclonal antibody diluted at 1:100 dilution in incubation buffer followed by incubating in Alexa Fluor® 488 goat anti-rabbit IgG antibody at a dilution of 1:1000 for 1 hour.

For p-eNOS and e-NOS the samples were also probed for 1 hour with p-eNOS and eNOS rabbit monoclonal antibody diluted at 1:100 dilution in incubation buffer followed by incubation in Alexa Fluor® 488 goat anti-rabbit IgG antibody at a dilution of 1:1000 for 1 hour.

At the end of the incubation period, the cells were centrifuged and the supernatant was removed the cells resuspended in 500µl PBS and analysed using flow cytometer (Becton Dickinson, Oxford, UK) on the FL1 channel to count 10,000 events (Vermes *et al*., 2002; Maddock *et al*.2002).

6.2.9 MTT cell viability assay using HL-60 cell line

Human leukaemia cancer cell lines HL-60 were obtained from the ECACC. The cells were cultured in RPMI 1640 media without L-Glutamine (Biosera, Ringmer, UK) which was supplemented with 10% fetal bovine serum, 2mM L-glutamine, HEPES and 0.1% antibiotic solution (100 U/ml penicillin, 0.1mg/ml streptomycin from Invitrogen). The cells were maintained at 37°C under a humidified atmosphere and 5% CO_2. 50µL of the cells containing ($1x10^5$cells/well) were plated in 96-well flat-bottomed microtitre plates, and treated with varying concentrations of artemisinin (0.4mM), doxorubicin (1µM), artemisinin (0.4mM) ± doxorubicin (1µM), artemisinin (0.4mM) ± doxorubicin (1µM) ± L-

NAME (100µM), doxorubicin (1µM), artemisinin (0.4mM) ± doxorubicin (1µM) ± aminoguanidine (100µM) for 24 hours. At the end of the incubation period, the cells were terminated by adding MTT solution (20µl of 5mg/ml MTT to each well) and further incubated for 2 hours at 37°C. 100µl of lysis buffer (20% sodium dodecyl sulfate in 50% dimethyl formamide) was then added to each well containing treatment and control samples.

Incubation was at 37°C and its duration was based on results of 24 hours assay optimization experiments from previous experiments in the lab (data not presented in this study). The plates were incubated overnight to solubilise the cells. The absorbing intensity of each well was determined at 492nm using a plate reader (Anthos 2001) which measures the percentage of cell growth calculated using the following formula;

% Cell Growth = (average absorbance of treated wells for Artemisinin/average absorbance of untreated control wells) x 100%.

Each experiment was repeated three times and the values estimated using the 4-parametric logistic analysis (Grafit Software, Erithacus, UK). Dose–response was plotted for artemisinin, doxorubicin and the combination of the two using inhibitors.

6.2.10 Nitric oxide Assay using the OxiSelect™ In Vitro Nitric Oxide Calorimetric Assay

In this assay, the nitrate (NO_3^-) in the sample is converted to nitrite (NO_2^-) by nitrate reductase enzyme which is absorbed at 540nm according to manufacturer's instructions

6.2.10.1 Experimental protocol using the Nitric Oxide assay

Adult ventricular rat myocytes were isolated from Sprague Dawley rats by enzymatic dissociation method as described previously in chapter 2. The isolated myocytes were counted using a using a nucleo counter (Chemometec, Sartorius, Surrey, UK) and resuspended in restoration buffer (RB) to a density of 100,000 cells/ml.

Preparation of Samples and standards were according to manufacturer's instructions. Nitrite and nitrate standards were prepared by making a dilution series of concentration range 0-

140µM from a standard 14mM of the nitrite. Triplicate wells were used for measuring the nitrate concentration.

6.2.10.2 Measurement of Total/Nitrate levels

Nitrate levels in the samples were measured by subtracting nitrite only from total nitrite and nitrate.

Nitrate = (Total nitrite + nitrate) - Nitrite only.

To measure the nitrite levels, 50µL of nitrite standards, samples or blanks were added to a 96 well plate. Followed by 50µL of PBS and 50µL of Griess Reagent A were added to each well. The plate was then incubated for 10 minutes allowing the colour to develop. Absorbance was then read at 540nm on a microplate reader. The concentration of nitrite was calculated by comparing sample absorbance to standards. Negative controls without nitrate were subtracted from reading. Each nitrite standard and sample was assayed in duplicate.

6.2.10.3 Measurement of Total/Nitrate via Nitrate Reduction

To measure nitrate levels, 50µL of nitrate standard, samples or blanks to the 96 well plate. Drug treated cells were subjected to artemisinin (4.3µM) and/or doxorubicin (1µM) treatment. The enzyme reaction mixture was also prepared for the number of tests to be performed. The diluted enzyme cofactor was added to the mixture (1:100 dilution in deionized water) and vortexed. 50µL of the enzyme reaction mixture was then added to the wells containing the sample/nitrate and covered with foil. The plates were then incubated for 1 hour at room temperature on an orbital shaker. 50µL of Greiss Reagent A was also added and vortexed followed by Greiss Reagent B. The plate was then incubated for a further 10 minutes for colour development absorbance was then read at 540nm. The sample's absorbance was then calculated by comparing the Nitrate and standard curve and comparing our drug treatment to control.

Statistical Analysis

Results were expressed as Mean ± SEM for infarct/risk ratio, cell viability, cleaved caspase-3 analysis, iNOS, p-eNOS $_{(Ser\ 1177)}$ and nitric oxide assay. Infarct size was tested for group differences using one way ANOVA with LSD post hoc tests. P values of $P<0.05$ were

considered statistically significant. Haemodynamics: LVDP, HR and CF were assessed for statistical difference using two way ANOVA at the different time points.

6.3 RESULTS

6.3.1 Exclusion Criteria

Three rat hearts were excluded from this experimental study; two due to low viability of live cells following isolation (≤70%) and the other due to a tear in the ventricular wall while tightening the snare during ischaemia. All other groups have been included.

6.3.2 Haemodynamics

LVDP, HR and CF were assessed for statistical difference using two-way ANOVA at the different time points.

In the doxorubicin (1μM) treated hearts there was a significant decrease in LVDP compared to time matched I/R control hearts, a marked decreased was seen at 145 minutes (73.9±3.5% vs. 52.0±2.2%, $P<0.01$) and at 175 minutes (70.7±2.9% vs 50.7±3.5%, $P<0.01$ in doxorubicin treatment groups, Figure 52). There was no significant effect on HR and CF when comparing the different treatment groups.

Hearts treated with artemisinin (4.3μM) showed no significant increase in LVDP compared to I/R control as shown in chapter 3. However, it can be seen that artemisinin co-treatment with doxorubicin artemisinin reversed doxorubicin induced decreases in LVDP. At 145 minutes into reperfusion treatment administration or artemisinin with doxorubin significantly reversed doxorubicin induced decreases in LVDP add time point (72.0±5.2% vs. 52.0±2.2% in doxorubicin treatment, $P<0.01$, Figure 52) this effect remained significant until the experiment was terminated at 175 minutes (70.7±4.5% vs. 50.7±3.5%, $P<0.01$, Figure 52).

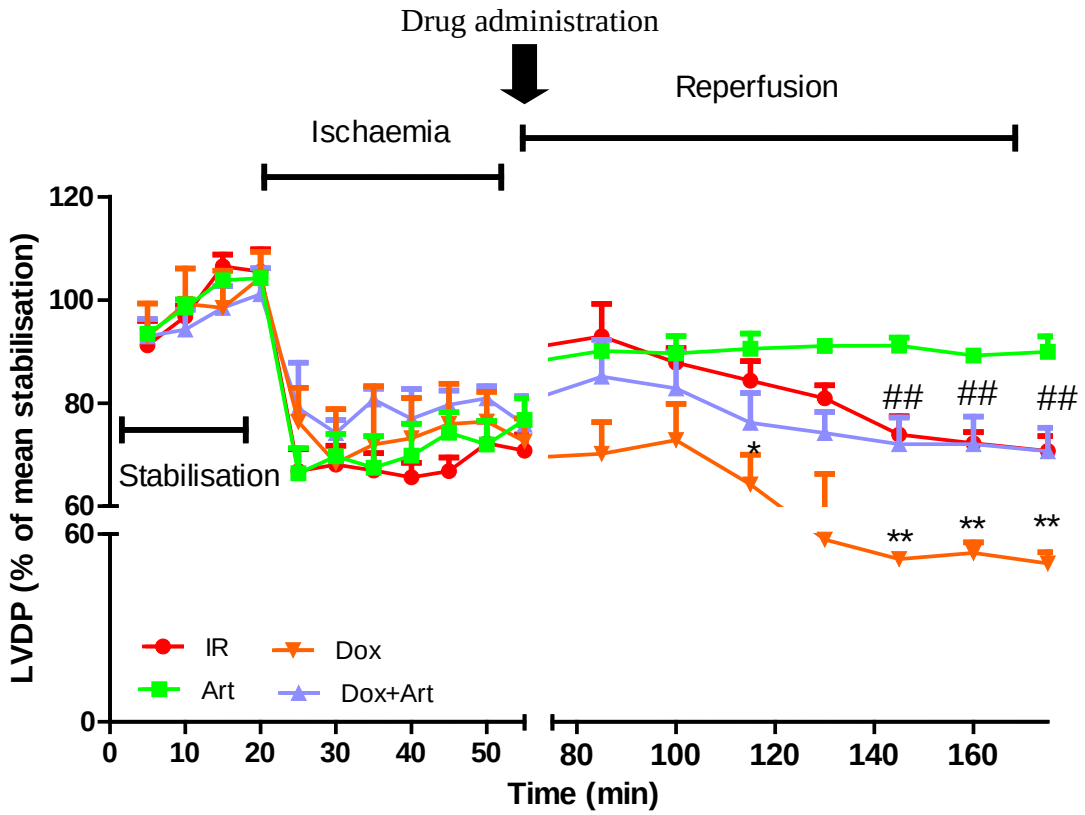


Figure 52*. The effects of artemisinin, doxorubicin and the co-administration of artemisinin and doxorubicin on LVDP. Hearts underwent 20 minutes of stabilisation, 35 minutes of ischaemia and 120 minutes of reperfusion, during which the drugs were administered at the onset of reperfusion. Data is expressed as Mean±sem. **P<0.01 vs. I/R control, *P<0.05 vs. I/R, #P<0.05 vs. Dox, ##P<0.05 vs. Dox (n=4-8)*

6.3.3 Treatment using artemisinin and doxorubicin confers protection from I/R and against doxorubicin induced injury.

Artemisinin (4.3µM) administered throughout reperfusion significantly decreased infarct size to risk ratio compared to the I/R control (38.0±2.5% vs. 55.8±1.7%, P<0.001, Figure 53). Doxorubicin administered at reperfusion significantly increased infarct size to risk ratio compared to the I/R control (71.3±3.2% vs. 55.8±1.7%, P<0.001, Figure 53)

Co-administering artemisinin (4.3µM) with doxorubicin (1µM) significantly reversed doxorubin mediated increase in infarct size to risk ratio (%) (45.7±2.5% vs. 71.3±3.2%, P<0.001, Figure 53).

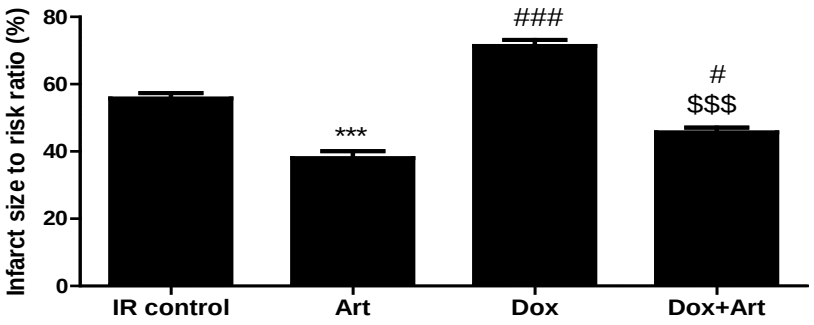


Figure 53. *The effects of doxorubicin (1μM) in the presence/absence of artemisinin (4.3μM) administered at reperfusion in an isolated perfused heart model subjected to I/R. Results are shown as Mean±SEM. ***P<0.001 vs. IR, ###P<0.001 vs. Art, #P<0.05 vs. Art, $$$P<0.001 vs. Dox. (n=3-8)*

To elucidate the mechanistic basis of artemisinin induced protection against the doxorubicin-induced cardiotoxicity earlier established in our ischaemia reperfusion studies, we also evaluated the effect of the nitric oxide cell survival pathway in cardiomyocytes subjected to H/R injury.

6.3.4 Artemisinin reverses doxorubicin and H/R induced injury in isolated cardiomyocyte via the activation of the nitric oxide cell survival pathway.

Cardiomyocytes previously isolated were subjected to 2 hours of hypoxia and 2 hours of reoxygenation and the drug treatments were administered throughout the reoxygenation period.

Treating the isolated cardiomyocytes with artemisinin (4.3μM) upon reoxygenation significantly improved cellular viability compared to the H/R group (66.5±6.3% vs. 29.3±6.1 % respectively, P<0.01, Figure 54). However, upon administering doxorubicin (1μM) during reoxygenation following hypoxia cellular viability was decreased by a further 10% compared to H/R however this did not reach statistical significance (19.2±2.4% vs. 29.3±6.1 % respectively, P>0.05, Figure 54).

Co-administration of artemisinin (4.3μM) with doxorubicin (1μM) upon reoxygenation significantly reversed doxorubicin mediated cytotoxicity (48.2±4.8% vs. 19.2±2.4%, P<0.001, Figure 54).

However, to determine the role of nitric oxide signalling in artemisinin mediated cytoprotection against doxorubicin induced injury in cardiomyocytes, we co-administered the non-specific nitric oxide synthase inhibitor L-NAME (100µM) in combination with artemisinin (4.3µM) and doxorubicin (1µM). Co-administering artemisinin (4.3µM) and doxorubicin (1µM) in the presence of L-NAME significantly increased viability compared to the artemisinin + doxorubicin group (72.0±0.7% vs. 48.2±4.8%, ($P<0.01$), Figure 54). Whereas comparing the results obtained from artemisinin + doxorubicin + L-NAME (100µM) group compared to doxorubicin alone (1µM) similarly showed significance (72.0±0.7% vs. 19.2±2.4%, $P<0.001$, Figure 54,). However, L-NAME (100µM) administered alone did not have an effect on myocyte viability compared to H/R control (47.0±7.1% vs. 29.3 ± 6.1% respectively, Figure 54).

Upon treatment with the specific iNOS inhibitor aminoguanidine (100µM), results showed a significant increase in cardiomyocytes viability compared to H/R control (46.4 ± 6.4 % vs. 29.3±6.1% respectively, $P<0.05$, Figure 54).

Interestingly, aminoguanidine (100µM) when administered in combination with artemisinin (4.3µM) and doxorubicin (1µM) during reoxygenation also showed a significant improvement in viability compared to doxorubicin treatment alone (43.5±7.1% vs. 19.2±2.4%, $P<0.01$, Figure 54). However, the combination of aminoguanidine (100µM), artemisinin (4.3µM) and doxorubicin (1µM) compared to artemisinin (4.3µM) alone was found not to be significant (43.5±7.1% vs. 48.1± 4.8%, Figure 54).

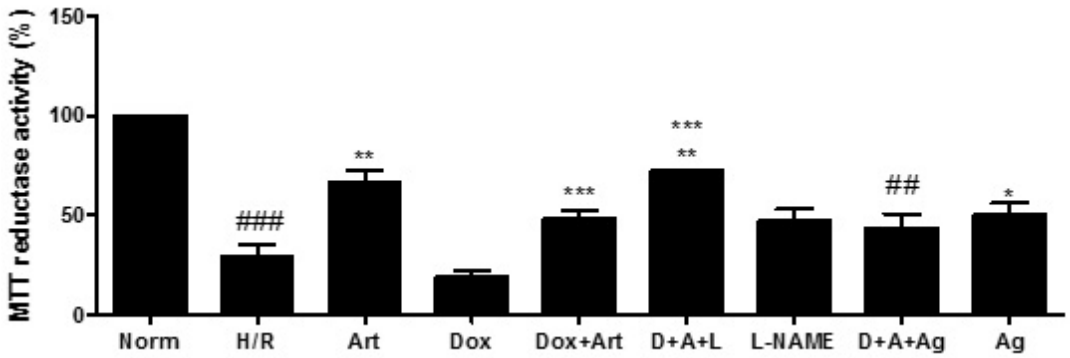


Figure 54: *MTT reductase activity used in assessing cellular viability in isolated ventricular cardiomyocytes subjected to two hours of hypoxia and two hours of reoxygenation. Dox (1µM) was administered in the*

*presence/absence of Art, L-NAME (100µM) and aminoguanidine (100µM). ### P<0.001 vs. Norm, **P<0.01 vs. HR, *P<0.05 vs. Art, ***P<0.001 vs. Dox, **P<0.05 vs. L-NAME, ##P<0.01 vs. Dox+Art (n=3-8) (D+A+L=Dox+Art+L-NAME and D+A+Ag= Dox+Art+Ag) (n=3-6)*

Results obtained from the myocyte cell viability assay, were used to evaluate the involvement of nitric oxide pathway in doxorubicin mediated cytotoxicity. Doxorubicin (1µM) was administerd in the presence of non selective NOS synthase inhibitor L-NAME or the selective iNOS inhibitor aminoguanidine.

Results showed doxorubicin (1µM) administered in the presence of L-NAME (100µM) had no significant effect on cellular viability compared to H/R (25.7±8.25 vs. 29.3±6.8% respectively, Figure 55). Similarly, doxorubicin (1µM) administered with L-NAME (100µM) compared to L-NAME was also not significant (25.7±8.25% vs. 47.0±7.1% respectively, Figure 55).

Cardiomyocytes were treated with doxorubicin (1µM) were also investigated in the presence of the iNOS inhibitor aminoguanidine (100µM) which showed no significant difference in cellular viability compared to aminoguanidine (100µM) treatment alone (45.7±8.1% vs. 50.1±7.4%, Figure 55). Cells treated with doxorubicin (1µM) and aminoguanidine (100µM) significantly reversed doxorubicin mediated decrease in cellular viability compared to doxorubicin treatment alone (45.7±8.1% vs. 29.3±6.1%,P<0.01, Figure 55).

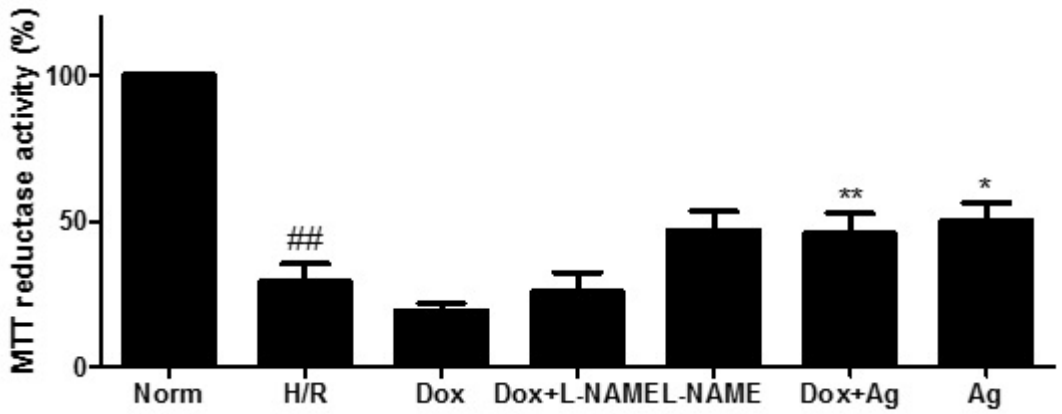


Figure 55. *The effects of doxorubicin and inhibition of the nitric oxide pathway on cellular viability in cardiomyocytes. Doxorubicin (1μM) was administered alone in the presence and absence of L-NAME (100μM) or aminoguanidine (100μM). ## P<0.001 vs. Norm, **P<0.01 vs. Dox, *P<0.05 vs. HR. (n=3-6).*

6.3.5 Artemisinin reverses doxorubicin mediated increases in cleaved-caspase-3

We assessed the levels of cleaved caspase-3 activity via flow cytometric analysis in isolated cardiomyocytes subjected to 2 hours of hypoxia and 2 hours of reoxygenation. Drug treatments were administered throughout reoxygenation as follows: artemisinin (4.3μM) alone, doxorubicin (1μM) treatment alone, artemisinin (4.3μM) ± doxorubicin (1μM), artemisinin (4.3μM) ± doxorubicin (1μM) ± L-NAME (100μM), artemisinin (4.3μM) ± doxorubicin (1μM) ± aminoguanidine (100μM).

By administering artemisinin (4.3μM) during reoxygenation, there was a significant decrease in cleaved caspase-3 activity compared to the H/R group (17.1±2.0% vs. 26.8±2.0% respectively, P<0.05, Figure 56). However, treatment with doxorubicin alone (1μM) significantly increased the levels of cleaved caspase 3 compared to H/R (48.0±2.15% vs. 26.8±2.0% respectively, P<0.001, Figure 56).

Co-administration of artemisinin (4.3μM) with doxorubicin (1μM) however significantly reversed the increase in cleaved caspase 3 activity observed in the doxorubicin treated group (24.0±2.5% vs. 48.0±2.15%, P<0.001, Figure 56).

Whereas by administering the combination of artemisinin (4.3μM) with doxorubicin (1μM) in the presence of the inhibitor of NOS, L-NAME (100μM), the results showed a reversal in the anti-caspase 3 activity of artemisinin, shown as an increase in cleaved caspase-3 levels compared to artemisinin treatment alone (33.0±1.4% vs. 17.14±2.0%, P<0.01, Figure 56). The combined treatment with artemisinin (4.3μM) with doxorubicin (1μM) in the presence of the inhibitor of NOS, L-NAME (100μM) compared to doxorubicin alone a showed significant decrease (33.0±1.4% vs. 48.0±2.15%, P<0.001, Figure 56).

Co-treating with a combination of artemisinin (4.3μM) with doxorubicin (1μM) in the presence of aminoguanidine (100μM) compared to artemisinin reversed the effect (4.3μM) (30.7 ± 1.1 % vs. 17.1 ± 2.0 %, P<0.01, Figure 56). Treatment with L-NAME showed, L-NAME had no significant effect compared to H/R control (29.9±0.7% vs. 26.8±2.0%, P>0.05, Figure 56). No significant effect was observed with aminoguanidine administered

alone throughout reoxygenation too compared to H/R control (28.0±2.1 vs. 26.8±2.0%, P>0.05, Figure 56).

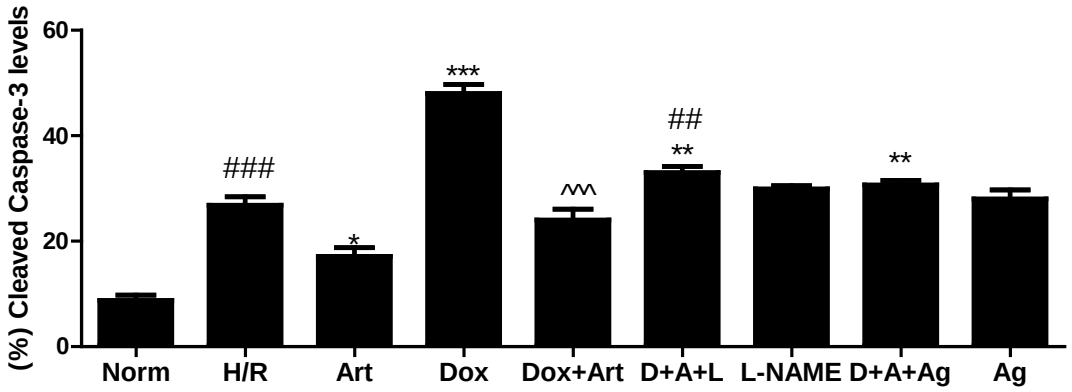


Figure 56. *Effect of administering doxorubicin (Dox) (1µM), artemisinin (Art) (4.3µM), both drugs combined and in the presence and absence of L-NAME (100µM) and aminoguanidine (Amg) (100µM) on cleaved caspase-3 activity in isolated adult rat cardiomyocytes after two hours of hypoxia followed by two hours of reoxygenation (HR) as analysed using Flow Cytometry. Results are shown as Mean±SEM. ***P<0.001 vs. Norm, *P<0.05 vs. HR, ***P<0.001 vs. HR, ^^^P<0.001 vs. Dox, **P<0.01 vs. Art (n=4-6). (D+A+L=Dox+Art+L-NAME) and (D+A+Ag=Dox+Art+Aminoguanidine).*

6.3.6 Effect of cleaved caspase-3 in isolated cardiomyocytes treated with doxorubicin alone and their involvement with nitric oxide pathway.

As described in section 6.3.6 doxorubicin (1µM) administration significantly increased cleaved caspase 3 activity compared to non-treated H/R control (48.0±2.15% vs. 26.8±2.0% respectively, P<0.001, Figure 56). We therefore further analysed the involvement of doxorubicin in nitric oxide signalling in relation to their effect on cleaved caspase-3 activity in isolated adult cardiomyocytes. Doxorubicin (1µM) administered with L-NAME (100µM) showed no significant difference compared to doxorubicin alone (34.3±8.7% vs. 48±2.1%, Figure 57). L-NAME alone partially reversed the combined effect of doxorubicin (1µM) with L-NAME (100µM) but did not reach significance (29.9±0.7% vs. 34.3±8.7%, Figure 57) and neither did it have an effect on cleaved caspase-3 activity (29.9±0.7% vs. 26.8±2.0%, Figure 57).

Co-treating the cardiomyocytes with doxorubicin (1µM) and aminoguanidine (100µM) was partialy reversed by aminoguanidine (100µM) but did not reach significance (36.7 ± 5.2 % vs 28.0 ± 2.1 %, Figure 57).

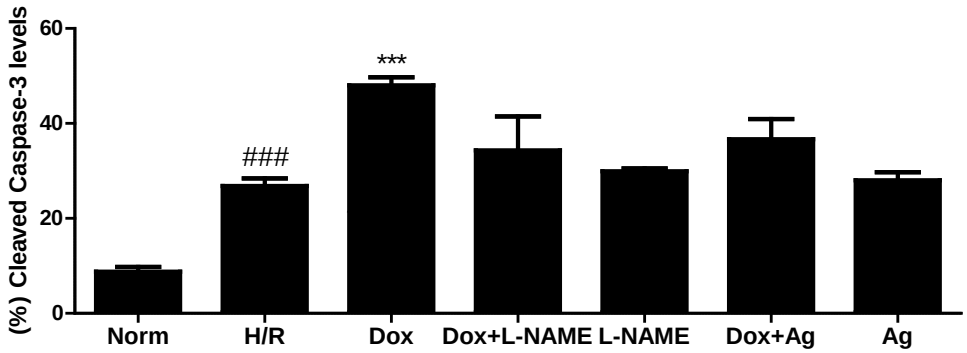


Figure 57. *Effect of doxorubicin (Dox) (1µM), artemisinin (Art) (4.3µM), both drugs combined and in the presence and absence of L-NAME (100µM) and aminoguanidine (Ag) (100µM) on cleaved caspase-3 activity in isolated adult rat cardiomyocytes after two hours of hypoxia followed by two hours of reoxygenation (HR) as analysed using Flow Cytometry. Results are shown as MEAN±SEM. ###P<0.001 vs. Norm, ***P<0.001 vs. HR. (n=4-6).*

6.3.7 Co-treatment with artemisinin (4.3µM) and doxorubicin (1µM) against doxorubicin (1µM) induced cytotoxicity in isolated cardiomyocytes shows involvement of eNOS (Ser 1177) as analysed using FACS analysis.

Isolated ventricular cardiomyocytes were subjected to 2 hours hypoxia and 2 hours reoxygenation with artemisinin (4.3µM) treatment to determine the levels of p-eNOS expression using FACS analysis. Untreated cardiomyocytes subjected to H/R showed a decrease in the expression of p-eNOS compared to the untreated normoxic control (46.3±2.0% vs. 100.0±0%, P<0.001, Figure 58a). Upon treating the cardiomyocytes with artemisinin, a significant (P<0.001) increase in the level of p-eNOS compared H/R control was observed (156.3± 31.0% vs. 46.3±2.0% respectively, Figure 58a). Also administering artemisinin (4.3µM) significantlly (P<0.001) increased the level of p-eNOS compared H/R control (156.3± 31.0% vs. 46.3±2.0% respectively, P<0.001, Figure 58a).

Administration of doxorubicin (1µM) with artemisinin (4.3µM), significantly increased levels of eNOS $_{(Ser\ 1177)}$ compared to doxorubicin treatment alone (223.0±11.35% vs. 63.5±7.2%, P<0.001, Figure 58a).

Treatment with artemisinin (4.3µM), doxorubicin (1µm) and a non-selective inhibitor of NOS, L-NAME (100µM), upon reoxygenation showed a significant decrease compared to L-NAME treated cardiomyocytes (149.7±23.2% vs. 51.0±10.6%, P<0.001, Figure 58a). Administering artemisinin (4.3µM), doxorubicin (1µm) and a non-selective inhibitor of NOS, L-NAME (100µM) showed a partial decrease in eNOS $_{(Ser\ 1177)}$ expression levels compared to artemisinin (4.3µM) and doxorubicin (1µm) but this did not reach statistical significance (149.7±23.2% vs 223.0±11.35% respectively, P>0.05, Figure 58a).L-NAME alone decreased expression of compared to H/R although it did not reach statistical significance (51.0±10.6% vs. 46.3±2.0%, Figure 58a).

Isolated cardiomyocytes treated with artemisinin (4.3µM), doxorubicin (1µm) and a non-selective inhibitor of NOS, L-NAME (100µM) showed a significant increase in eNOS$_{(Ser\ 1177)}$ expression levels compared to untreated H/R control (149.7±23.2% vs. 46.3±2.0% respectively, P<0.01, Figure 58a).

The combination of doxorubicin and artemisinin treated cardiomyocytes expressed a partial increase in the expression of eNOS$_{(Ser\ 1177)}$ which did not reach statistical significance compared to artemisinin treatment (223.0±11.35% vs. 156.3±31.0% respectively, P>0.05, Figure 58a).

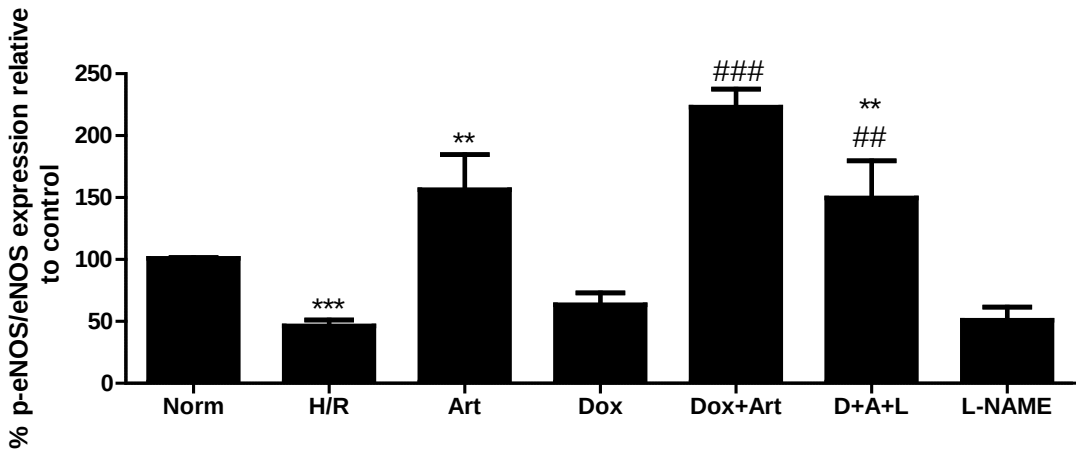


Figure 58a. *Effect of administering doxorubicin (Dox) (1µM) and artemisinin (Art) (4.3µM) throughout reoxygenation in the presence and absence of L-NAME (100µM) on eNOS (Ser 1171), expressions as analysed using Flow Cytometry. ***P<0.001 vs. Normoxia (Norm), **P<0.01 vs. HR, ###P<0.001 vs. Dox, ##P<0.01 vs. L-NAME (n=4-6) (D+A+L=Dox+Art+L-NAME).*

6.3.8 Effect on p-eNOS expression in cardiomyocytes subjected to H/R injury followed by doxorubicin treatment in the presence/absence of NOS inhibitor L-NAME

p-eNOS expression was significantly down regulated in the H/R group compared to normoxic control (46.3±2.0% vs 100.0±0%, P<0.001 respectively, Figure 58c). Administration of Doxorubicin (1uM) throughout reoxygenation had no significant effect on p-eNOS expression compared with H/R control group (63.5 ± 11.5% vs. 46.3±2.0%, P>0.05, Figure 58c).

Co-administration of doxorubicin (1µM) with L-NAME (100µM), also expressed similar levels of p-eNOS (Ser 1177) compared to doxorubicin alone when analysed using FACS analysis (63.3±15.2% vs. 63.5 ± 11.5%, P>0.05, Figure 58b). L-NAME (100µM), alone showed no significance in p-eNOS expression compared to H/R control (51.0±10.6% vs. 46.3±2.0%, P>0.05, respectively, Figure 58b).

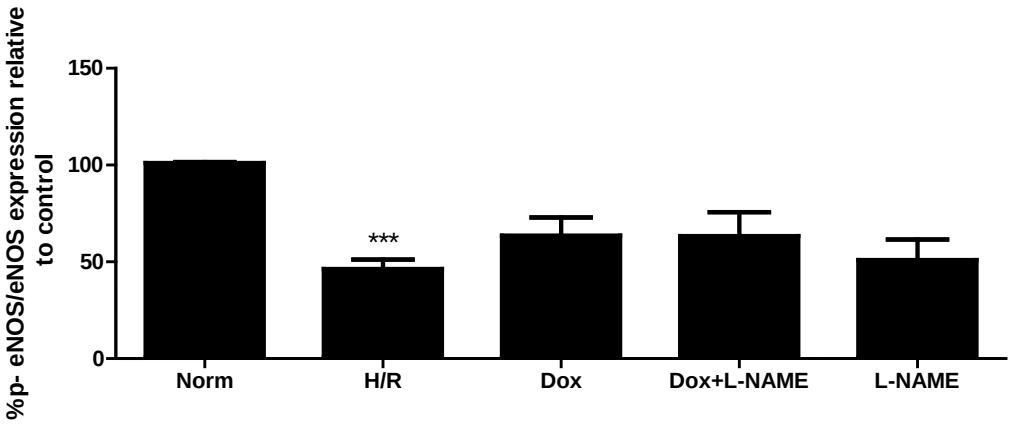


Figure 58b. *Effect of administering doxorubicin (1µM) throughout reoxygenation in the presence and absence of L-NAME (100µM) on eNOS expressions as analysed using Flow Cytometry. ***P<0.001 vs. Normoxia (Norm) (n=4-6).*

6.3.9 Doxorubicin used in combination with artemisinin in isolated cardiomyocytes subjected to H/R show elevation in iNOS expression with artemisinin and combination treatment.

Treatment with artemisinin (4.3µM) led to a significant increase in iNOS expression compared to H/R control (150.5±14.4% vs. 69.9±7.2%, respectively, P<0.01, Figure 59a). An increase in iNOS was also observed from using doxorubicin (1µM) and artemisinin (4.3µM) combined compared to H/R group (108.6±12.0% vs. 69.9±7.2%, P<0.01, respectively, Figure 59a).

6.3.10 Effect of iNOS expression upon treatment with a combination of doxorubicin and artemisinin in isolated cardiomyoctes subjected to HR in the presence/absence of L-NAME and aminoguanidine.

Adjunctive treatment with L-NAME showed significant upregulation in iNOS expression compared to L-NAME (83.6±7.4% vs 69.9±7.2%, P<0.05, Figure 59a). Treatment with doxorubicin (1µM) and artemisinin (4.3µM) and aminoguanidine (100µM) compared to doxorubin and artemisinin alone showed a significance decrease (76±5.4% vs. 108±12.0%, P<0.01, Figure 59a).

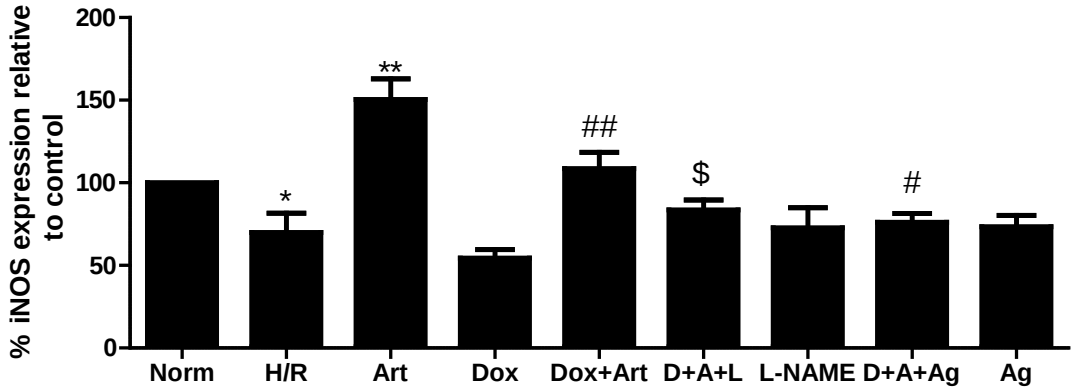


***Figure 59a**. Effect of doxorubicin (1µM) and artemisinin (Art) (4.3µM) on iNOS expression in the presence and absence of L-NAME (100µM) and aminoguanidine (Ag) (100µM) analysed using FACS analysis. *P<0.05 vs. Normoxia (Norm), **P<0.01 vs. HR, ##P<0.01 vs. Dox, $P<0.05 vs.L-NAME, #P<0.05 vs. Dox+Art. Data expressed as mean± SEM (n=4-6).*

6.3.11 Effect of iNOS expression in cardiomyocytes subjected to H/R followed by treatment with doxorubicin at reoxygenation in the presence/absence of L-NAME or aminoguanidine.

We also looked into the effect of doxorubicin on the nitric oxide pathway where we administered inhibitor L-NAME and aminoguanidine, as in the adjunctive treatment

Treatment with doxorubicin (1µM) alone compared to H/R showed a partial decrease in iNOS expression but this did not reach significance (54.4±6.9% vs. 69.9±7.2%, P>0.05, Figure 59b). Co-treatment of doxorubicin (1µM) with inhibitor L-NAME (100µM) also showed no significant effect compared to doxorubicin alone (1µM) (68.2±9.8% vs. 54.4±6.3%, Figure 59b); neither did aminoguanidine treatment (67.5±4.9% vs. 54.4±6.3%, Figure 59b).

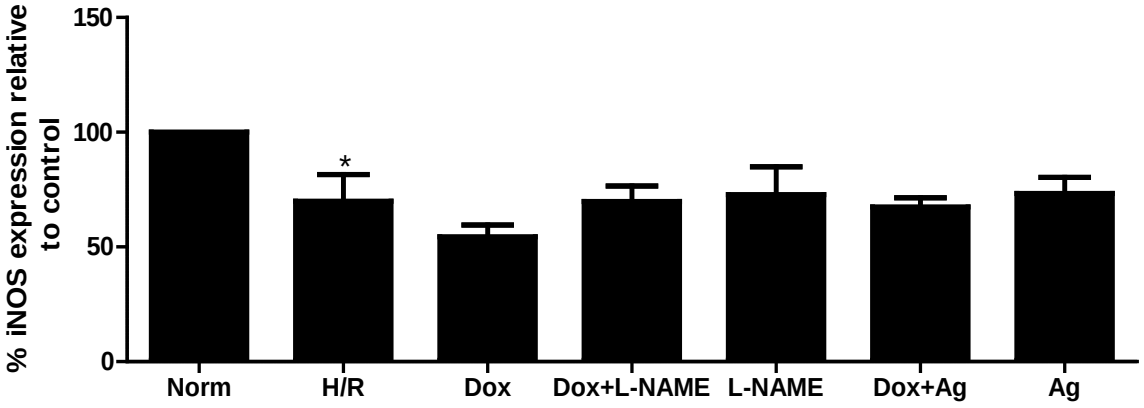


Figure 59b. *Effect of doxorubicin (1µM) on iNOS expression in the presence and absence of L-NAME (100µM) and Aminoguanidine (Ag) (100µM) analysed using FACS analysis. *P<0.05 vs. Normoxia (Norm) (n=4-6)*

6.3.12 Involvement of nitric oxide in doxorubicin induced cytotoxicity in HL-60 cells and attenuation it's via artemisinin administration.

In chapter 3, we established that artemisinin (0.4mM) possesses cytotoxic properties against HL-60 cells. In order to investigate the effect of co-administering artemisinin (0.4mM) and doxorubicin (1µM) in HL-60 cells, we used MTT analysis and compared the drug treatment groups to the control. Both artemisinin and doxorubicin administered alone were significantly cytotoxic against HL-60 cells, with artemisinin showing (58.6±2.0% vs. 100±0%, P<0.001) and doxorubicin (43.2±8.6% vs. 100%±0%, P<0.001, Figure 60) compared to control. Co-treatment with artemisinin (0.4mM) nearly doubled doxorubicin's (1µM) anti-cancer properties (28.2±3.6% vs. 43.2±8.6%, P<0.001, Figure 60). We further used the non-selective inhibitor of nitric oxide L-NAME (100µM) against HL-60 cells: L-NAME (100µM) alone did not cause any effect compared to control (97.7±4.7% vs. 100±0%). Using L-NAME (100µM) together with a combination of artemisinin (0.4mM) and doxorubicin (1µM) significantly (P<0.05) increased cytoxicity compared to artemisinin (71.7±6.1% vs. 58.6±2.0%) or (71.7±6.1% vs. 43.2±8.6%, P<0.05) compared to doxorubicin treatment. L-NAME blocked the observed effect of artemisinin, co-treated with doxorubicin and L-NAME (71.7±6.1% vs. 97.7±6.6%, P<0.05, Figure 60). Comparing doxorubicin and artemisinin treated HL-60 to L-NAME also showed significance (28.2 ±3.6% vs 97.7±6.6%, P<0.001, Figure 60)

6.3.13 Effect of adjunctive treatment with inhibitors (L-NAME and aminoguanidine) in HL-60 cells analysed using MTT reductase activity.

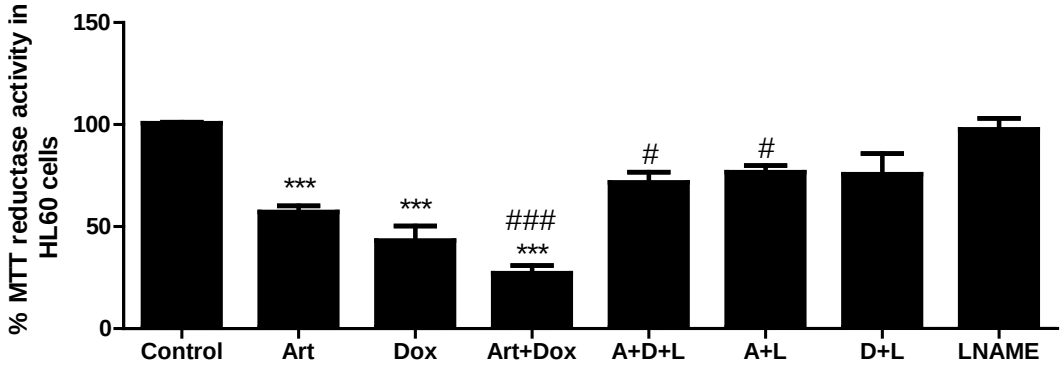


Figure 60. *The effects of artemisinin (0.4mM), doxorubicin (1µM), L-NAME (100µM) and various combinations of the three drugs on the cytotoxicity of HL-60 cancer cells. ***P<0.001 vs. control, #P<0.05 vs. Art/Dox, ###<0.001 vs L-NAME Data presented as mean ±SEM (n=4)*

	MTT		**iNOS**		**eNOS**		**Caspase-3**		**HL-60**	
Norm	100 ± 0 %		100 ± 0 %		100 ± 0 %		8.8 ± 1.2 %		100 ± 0 %	
H/R	29.3 ± 6.1 %	*** vs. Norm	69.9 ± 13.5 %	* vs. Norm	46.3 ± 2.0 %	*** vs. Norm	26.8 ± 2.0 %	*** vs. Norm		
Art	66.5 ± 6.3 %	** vs. H/R	150.4 ± 14.4 %	** vs. Art	156.3 ±31.0 %	*** vs H/R	17.1 ± 2.0 %	* vs. H/R	57.2 ± 2.9 %	*** vs. Norm
Dox	19.2 ± 2.8 %	NS vs. H/R	54.4 ± 6.3 %	NS vs. H/R	63.5 ± 11.5 %	NS vs. H/R	48.0 ± 2.1 %	*** vs. H/R	43.2 ± 8.6 %	*** vs. Norm
Dox + Art	48.2 ±4.8 %	*** vs. Dox	108.6 ± 12.0 %	** vs. H/R	223 ± 17.9 %	*** vs. Dox	24.0 ± 2.1 %	*** vs. Dox	27.2 ± 4.6 %	*** vs. Dox
Dox +Art +L-NAME	72 ± 0.7 %	*** vs.Dox ** vs. Art+Dox	83.6±7.4%	* vs. L-NAME	149.7 ± 36.9 %	** vs L-NAME	33.0 ± 1.4 %	** vs. Art	71.7 ± 6.1 %	*** vs. L-NAME * vs. Dox/Art
L-NAME	47 ± 7.1 %	NS vs H/R	72.8 ± 14.0 %	NS vs. H/R	51.0 ± 10.6 %	NS vs. H/R	29.9 ± 0.7 %	* vs. H/R	97.7 ± 6.6 %	NS vs. H/R
Dox+Art+Amg	43.5 ± 7.1 %	** vs. Dox	76.1 ± 5.4 %	** vs. Dox+Art			30.7 ± 1.1 %	** vs. Art		
Amg	46.4 ± 6.4 %	* vs H/R	73.3 ± 8.0 %	NS vs. H/R			28.0 ± 2.1 %	NS vs. H/R		
Art + L-NAME	48.1 ± 5.5 %	* vs. Art	71.5 ± 5.8 %	** vs. Art	59.6±8.1%	** vs. Art	31.0 ± 1.4 %	** vs. Art	76.7 ± 4.0 %	* vs. L-NAME
Dox+L-NAME	25.7 ± 8.2 %	NS vs. H/R	70.0 ± 8.7 %	NS vs. Dox	63.3 ± 15.2 %	NS vs. Dox	34.3 ± 8.7 %	NS vs. L-NAME	75.7 ± 12.4 %	NS vs. L-NAME
Dox+Amg	45.7 ± 6.3 %	** vs. H/R	67.5 ± 4.9 %	** vs H/R			36.7 ± 5.2 %	*** vs. Art		

Table 5. *Summary table showing the effect of adjunctive treatment with inhibitors (L-NAME and aminoguanidine) in cardiomyocytes and in HL-60 cells analysed using MTT reductase activity, FACS analysis and caspase 3 assay. *P<0.05, **P<0.01 and ***P<0.001. Results are expressed as MEAN±SEM*

6.4 DISCUSSION

Despite the advances in chemotherapy, presently administered anticancer therapies are not without their complications (Volkova and Rusell 2011). One of the most common therapeutic anticancer agent is the anthracyclines, with doxorubicin being the most effective and frequently administered anthracycline (Rahman *et al*., 2007). The effect of doxorubicin that has been observed clinically and is not limited to tumour cells only; this is because doxorubicin's efficacy in treating cancer is limited by its cumulative dose-dependent cardiotoxicity (Volkova and Rusell 2011).

Deleterious effects of doxorubicin were also observed from our haemodynamic effects, where doxorubicin significantly decreased LVDP during reperfusion in isolated hearts. Our findings are in concordance with previous studies that have also associated the drop in LVDP with administering doxorubicin (Cai *et al*., 2010; Ramond *et al*., 2008; Gharanei *et al*., 2014). Concurrent administration of artemisinin at reperfusion significantly reversed the myocardial changes in terms of both haemodynamics (LVDP) and the cellular changes induced by doxorubicin treatment.

Doxorubicin has been shown to affect various tissues/cells other than the targeted tumour cells, which may lead to serious consequences to the healthy tissues in other organs. Some of the documented effects vary from bone marrow toxicity, which causes cells to rapidly divide, to more serious cellular apoptosis/necrosis (Minotti *et al.,* 2004). Cardiac myocytes, when affected by doxorubicin toxicity, can lead to serious consequences due to their limited capacity to regenerate (Minotti *et al.,* 2004). Cardiac apoptosis induced by doxorubicin has been related to the production of excessive reactive oxygen species by the mitochondrial NADPH dehydrogenase (Gilleron *et al*., 2009). Doxorubicin treatment of HL-60 has been observed to trigger increases in caspase-3-like activity, which is a common feature of apoptosis (Crespo-Ortiz and Wei 2012; Childs *et al*., 2002).

Doxorubicin induced cardiotoxicity has been linked to an increase in cleaved caspase-3 activity as shown in our study as well as in previous studies (Khan *et al.*, 2006). In addition to this, Khan *et al*. (2006) also showed an increase in the expression of Bax protein and the release of cytochrome c which are known to mediate cell death. This supports our findings

of infarct size increasing with doxorubicin treatment and increase in activated caspase-3, which is a known marker for apoptosis. Doxorubicin's cytotoxicity has been associated with the generation of free radicals, which cause lipid peroxidation, mainly in the cell membrane, leading to cell damage (Banfi *et al*., 1992; Fukuda *et al*., 1992). Our results show that doxorubicin induces more cardiotoxicity in isolated perfused rat hearts subjected to I/R compared to control and to H/R, which we suggest is via the manifestation of oxidative stress caused by the generation of free radicals. Gilleron *et al.* (2009) showed that doxorubicin administration was accompanied by increased superoxide production through NADPH oxidase activation in rat cardiomyoblasts. Inhibiting these enzymes leads to a reduction in doxorubicin-induced reactive oxygen species production and caspase-3 like activity (Gilleron *et al.,* 2009).

Doxorubicin treatment also greatly decreased cellular viability in the cardiomyocyte subjected to H/R injury. Mitochondria also plays a central role in the cardiotoxic effect of doxorubicin by regulating the implicating cytochrome c, Bax, caspase-3 and so on. Gharanei *et al.* (2014) also showed doxorubicin induced cardiotoxicity affcets mitochondrial activity and integrity which leads to mitochondrial dysfunction and eventually death. This was suggested to be as a result of the accumulation of ROS and calcium overload which leads to oxidative stress, causes a depletion in ATP, mitochondrial dysfunction (as a result of change in mitochondrial permeability), cellular necrosis and initiation of apoptosis (Halestrap *et al.,* 2004; Hausenloy *et al.,* 2002, Minotti *et al.,* 2004). Minotti *et al.* (2004) have shown that off-target effects of doxorubicin treatment can manifest into serious cardiovascular complications; however, the mechanisms of action still remain unknown, although they have been found to be multifactorial. Gharanei *et al.,* (2014) have identified one of the potential ways of attenuating doxorubicin-induced cardiotoxicity is by the inhibition of the mPTP which is a novel adjunctive therapy route to reducing the manifestations of doxorubicin-induced cardiotoxicity. We postulate that the use of an adjunctive drug regimen may be effective in reversing the doxorubicin induced cardiac toxicity in the isolated perfused heart as well as well as in cardiomyocytes. The effects of using the adjunctive therapy treatment will then be tested in a cancer cell line in order to investigate the effect of the co-treatment on anti-cancer properties of doxorubicin.

Previous research similarly administered C-phycocyanin, which protects against I/R injury and ameliorates doxorubicin induced oxidative stress and apoptosis in rat cardiomyoctes (Khan *et al*., 2006). Doxorubicin has been shown to induce DNA fragmentation, apoptosis, increase Bax expression, increase cytochrome c and also increase caspase-3 activity. The aforementioned deleterious effects were markedly attenuated by C-phycocyanin which was shown to ameliorate I/R induced cardiac dysfunction through its antioxidant and antiapoptotic actions and also via the modulation of p38 MAPK and ERK1/2 (Khan *et al*., 2006)

With the aim of reducing the known cardiotoxic effect of doxorubicin, this study used artemisinin as an adjuvant therapy. It is imperative to assess the effects in previous settings such as isolated perfused hearts, isolated cardiomyocytes subjected to I/R or H/R injury and also in cell viability/caspase-3 assays. The effects of adjunctive therapy in a cancer line were also established in order to investigate whether co-treatment with artemisinin will affect the cytotoxicity of doxorubicin against cancer cells. This study further supports the crucial role of artemisinin as a cardioprotective agent and its potential against doxorubicin induced cardiotoxicity.

With recent medical interventions and research development, cancer patients are able to survive much longer but with higher chances of developing or exacerbating other pre-existing conditions most popularly, cardiomyopathies (Civelleri *et al*., 2000). Previous studies as well as previous chapters have shown doxorubicin exacerbates I/R injury in both naïve and stressed hearts. By administering artemisinin, it was therefore necessary to investigate the off target effects of the potential adjuvant therapy both in naïve and diseased conditions (simulated reperfusion injury).

In our previous chapters, we have established that artemisinin's mediated cardioprotective effects is via the nitric oxide cell survival pathway. We therefore investigated whether artemisinin's protection against doxorubicin-induced toxicity is via the same pathway described earlier. Artemisinin has been shown to structurally possess an endoperoxide bridge and in antimalarial treatment, the endoperoxide bridge has been shown to play a key role in the inihibition of the parasite (Wang *et al.,* 2010). A study by Wang *et al*. (2010)

showed the accompanied dysregulation in the malarial mitochondria was responsible for impairing functions which was usually followed by a rapid burst in ROS levels. Rapid short burst of ROS are not necessarily deleterious, in a different setting of I/R injury, short burst of ROS generated from preconditioning (short cycles of ischaemia/reperfusion prior to the onset of ischaemia) has been shown to decrease infarction and resulted in an upregulation of potentially protective proteins in different modes including rats and pigs (Heusch *et al.*, 2008).

This chapter, however, the focus aim was to establish the effect of artemisinin in doxorubicin-induced cardiotoxicity via infarct analysis, their combined effect in cellular viability assays and in activated caspase-3 levels in cardiomyocytes, furthermore we it investigated the cell signalling pathway involved in the observed effect in the cardiomyocytes as well as upon co-administering artemisinin and doxorubicin in on HL-60 cells using MTT assay.

In the isolated perfused hearts, doxorubicin decreased haemodynamic functions and increased infarct to risk ratio in I/R tissue as shown in Figure 52. By administering artemisinin at reperfusion, artemisinin reverses this effect. Artemisinin has a dose dependent decrease in I/R and improves viability in the MTT assay. Our results also show an increase in nitric oxide synthesis via eNOS and iNOS synthesis with co-treatment using artemisinin and doxorubicin in cardiomyocytes.

Our results showed an increase in p-eNOS expression with adjunctive treatment**.** This data suggests that nitric oxide may be upregulated via the eNOS pathway with administering adjunctive treatment of artemisinin and doxorubicin. Upregulation in eNOS expression has been associated with cardioprotection in rat models of I/R injury (Felaco *et al*., 2000). p-eNOS was downregulated by co-treating the cardiomyocytes with doxorubicin, artemisinin and the non selective inhibitor, L-NAME resulting in a decrease in eNOS expression. This effect was implicated with adjunctive treatment as treating the cells with doxorubicin or L-NAME alone showed a down-regulation of eNOS expression. Previous studies have shown L-NAME decreases eNOS activity in several models including I/R models in rats, memory

models using rats and several cancer models (Boultadakis and Pitsikas 2010; Hussain *et al.*, 2013; Johansson *et al.*, 1998; Klamer *et al*., 2001)

Artemisinin has previously been been identified in telemore protection study using Swiss mice as an inhibitor of NOS and an inducer of nitric oxide (Zeng and Zhang, 2011; Zeng *et al.*, 2011). This supports our findings that artemisinin mediates protection via NO cell signalling pathway. Pharmacokinetic research has also shown that at concentrations of 50μM, artesunate (a derivative of artemisinin) simulated NOS induction and nitric oxide production in hepatoma cell line (Zeng and Zhang 2011). Concentrations above 100μM however, led to abrogation of nitric oxide generation (Zeng and Zhang 2011). In other studies however, artesunate was shown to inhibit nitric oxide synthesis in bacterial proliferation studies (Zeng *et al.*, 2011). In the present study, adjunctive treatment with doxorubicin and artemisinin implicated the nitric oxide pathway which was shown to be independent of doxorubicin treatment in our study. Interestingly, a study using human colon cancer HT29 cells showed NOS induction increases doxorubicin efficacy using simvastatin (sodha *et al.*, 2008).

However, another study using bovine aortic endothelial cells, contradicted our findings by by showing that doxorubicin-induced apoptosis is linked to the redox activation of doxorubicin by eNOS (Kalivendi *et al*., 2009). Kalivendi *et al.* (2009), however, explained that the cardiovascular toxicity of doxorubicin was solely attributed to the enzymatic switch in the activity of eNOS to a superoxide generating enzyme (i.e. NADPH oxidase activity). Vasquez-vivar *et al.* (1997) explained that this phenomenon of eNOS upregulation by doxorubicin (5μM) is a result of the reduction of doxorubicin reductase domain of eNOS observed to generate peroxynitrite and hydrogen peroxide, both potent oxidants that are implicated in several vascular pathologies.

Research has shown a difference in the nitric oxide generated in non-preconditioned/unstressed myocardium compared to nitric oxide in preconditioned myocardium, which explains the phenomenon whereby the heart displays a defensive phenotype in response to stress conditions (Bolli 2001). We also believe that there is a difference in the nitric oxide generation in artemisinin's cardioprotection and its

cytotoxicity. Bolli (2001) explained that eNOS has a well-established and immediate cytoprotective function, which is, however, short term, while iNOS has a delayed but long term defence function, which distinguishes protection in the unstressed/non-preconditioned myocardium and the late preconditioned myocardium. Our findings showing cytoprotection implicated the upregulation of iNOS expressions with adjunctive treatment. This was supported by Smart *et al*. who similarly showed pre-treating cardiomyocytes with interleukin-6 (IL-6) was cardioprotective via the PI-3 kinase and NO-dependent pathway of cytoprotection through the upregulation of iNOS (Smart *et al*., 2006).

In our studies, we assessed the cardiomyocytes for p-eNOS expression, where a non selective nitric oxide inhibitor L-NAME was administered in the study results showed an upregulation of over 4 fold increase in p-eNOS levels with adjunctive treatment compared to H/R control. Upregulation in eNOS expression as previously described is well established in cytoprotective agents (Bolli 2001). However, we also observed an increase in iNOS expression using combined drug treatment, with a nearly 2 fold increase compared to H/R control, administering aminoguanidine (a selective iNOS inhibitor aborageted the observed effect suggesting involvement of iNOS. Despite iNOS reported to have detrimental effects with iNOS knock out mice (mice were of C57BL/6 strain bred into the B6C3 background) shown to exhibit mitochondrial damage such as cytoplasmic swelling and degeneration of the mitochondria when compared to wildtype mice (Cole *et al*., 2006, Kang *et al.,* 1997). Studies have also reported slight increase in nitric oxide to show protection in adriamycin induced cardiotoxicity where adriamycin was shown to induce cardiac injury via the generation of superoxide (Cole *et al.,* 2006, Pacher *et al.,* 2003). In the study by Cole *et al*. (2006), structural improvements to the mitochondria as well as improvements in cardiac function and decrease in biochemical injury markers such as cardiac troponin, creatine phosphokinase, and lactate dehydrogenase in transgenic manganese superoxide dismutase (MnSOD) overexpressing animals crossed with iNOS null mic were observed. This suggested that nitric oxide produced by iNOS may contribute to protection of normal tissue by adriamycin induced injury *in vivo* which further suggests a slight increase in iNOS expression upon administering artemisinin and doxorubicin against doxorubicin induced injury is protective. Cole *et al*. (2006) further suggested that reduction in nitric oxide levels mediated by adriamycin treatment has led to an increase in cardiac mitochondrial injury can

be attenuated by a compensatory increase in MnSOD which supports increase in iNOS expression levels.

Furthermore studies using artemisinin have shown an upregulation in iNOS expression in the treatment for allergic asthma (Ho *et al.,* 2012). Majority of studies reviewed by Bolli (2001) also examined the role of nitric oxide *in vivo* and *in vitro* and concluded that both endogenous and exogenous nitric oxide have a protective role, with a small minority reporting a detrimental effect suggesting a ying-yang effect with nitric oxide upregulation (Palcher *et al.*, 2007; Bolli 2001). Upregulation of nitric oxide synthesis has been associated with a cytoprotective function in the heart, especially in preconditioning, where the heart attempts to protects itself (Bolli, 2007).

In the present study, we were able to show that artemisinin has the ability to salvage cardiomyocytes damaged as a result of H/R followed by doxorubicin treatment. The observed protection was linked with the nitric oxide-dependent pathway of protection, seen as an increase in iNOS and eNOS in isolated ventricular myocytes. Gene transfer of eNOS and iNOS have been shown to present infarct-sparing actions in ischaemic preconditioning models, thus implicating both in the protection of the heart against ischaemia/reperfusion injury (Bolli 2001).

Studies have postulated several different molecular mechanisms responsible for anthracycline induced cardiomyopathy, cardiotoxicity is mainly associated to redox recycling, ROS generation, a disturbance of Ca^{2+} homeostasis, altering nucleic acid by intercalation into DNA and modulate intracellular signalling, which may lead to disruption in homeostatic process, development of sacropenia or even cell death (Kim *et al.*, 2006; Zhang *et al*., 2012; Geisberg and Sawyer 2010; Chen *et al.,* 2007). Despite over four decades of investigating anthracylines, the pathogenic mechanisms responsible for their cardiotoxicity has not been completely elucidated (Gianni *et al.,* 2008). Anthracyclines structurally consist of a tetracyclic aglycone linked with an amino sugar. The quinone moiety of anthracycline could serve as an electron acceptor, which contributes to the production of ROS (Vejpongsa and Yeh, 2013). In studies using HL-60 cell lines, artemisinin has been shown to induce damage via the generation of ROS, which was found

to activate apoptosis (Michaelis *et al*., 2010). Doxorubicin induced free radical generation can interact with oxygen to form superoxide hydroxyl radicals which can lead to DNA damage (Eliot *et al.,* 1984; Benchekroun *et al*., 1993).

Our results showed artemisinin significantly improved cellular viability in cardiomyocytes, protected the myocardium against doxorubicin-induced cardiac injury and exacerbated doxorubicin's cytotoxicity against HL-60 cells. Previous research by Efferth *et al.* (2007) showed that artesunate (an artemisinin derivative) improved efficacy of doxorubicin against tumour cells, with the synergistic effect largely explained by the fact that artesunate and doxorubicin use different killing mechanisms. Doxorubicin has also been shown to stimulate nitric oxide production in Ehrlich ascites carcinoma (EAC) cells as well as in murine breast cancer cell line (Sayed-Ahmad *et al*., 2001; Bani *et al.,* 1995). These studies proposed nitric oxide may contribute to doxorubicin's antitumour activity in the two cell lines they have tested. Sayed-Ahmad et al. (2001) showed treatment with L-NAME or aminoguanidine (both inhibitors of nitric oxide) significantly decreased doxorubicin induced nitric oxide production and attenuated the effect on tumour cells (Sayed-Ahmad *et al.,* 2001). Furthermore, research by Haywood et al., (1996) and Vejlstrup et al.,(1998) showed that the high levels of nitric oxide was as a result of the inducible nitric oxide generated however, Sayed-Ahmad et al., (2001) in their study suggested the increased in nitric oxide is as a result of eNOS transcription in the EAC cells as shown in our results.

Artemisinin induces apoptosis in leukemic T cells mainly through the intrinsic mitochondrial pathway *via* generation of reactive oxygen species (ROS) while doxorubicin kills cancer cells via DNA intercalation (Efferth *et al*., 2007). In our results we observed adjunctive treatment with doxorubicin and artemisinin reversed the increase in caspase-3 activity with doxorubicin treatement. Treatment with doxorubicin alone increased the level of caspase-3 activity compared to control. This was similarly observed in previous studies investigating the apoptotic sensitivities of neonatal and adult mouse hearts. Doxorubicin was shown to induce myocardial apoptosis in both neonatal and adult cardiomyocytes with a dose dependent cardiotoxicity that was shown to be caspase dependent (Shi *et al.,* 2012).

In our studies using HL-60 cells, artemisinin was shown to be cytotoxic against HL-60 cells with its observed cytotoxicity implicating the nitric oxide pathway as shown in our MTT assay where treatment with artemisinin showed an effect that was reversed by the non selective NOS inhibitor, L-NAME.

This suggests that artemisinin in cardiomyocytes and in HL-60 cancer cells implicates the nitric oxide pathway in different ways. In our HL-60 assay, treatment with aminoguanidine was shown to have an effect on cytotoxicity thus did not inhibit nitric oxide expression in our study. Previous studies have reported hydroxyguanidine, thiosemicarbazide, and substituted benzohydroxamic acid all express promising antitumor activities by inhibiting DNA synthesis as a consequence of inhibiting ribonucleotide reductase (Saiho *et al.*, 2010).

HL-60 cell lines have extensively been used by our group and others to investigate the effect of different drug treatments. With adjunctive treatment, our results showed an increase in iNOS and eNOS with HL-60 cell line, investigating the cell cycle regulators, downstream of checkpoint kinase activation will have provided a greater insight into adjunctive treatment. Previous studies have reported that artemisinin markedly increases the degree of differentiation in HL-60 cells when combined with low doses of 1, α 25-dihydoxyvitamin D3 [1, 25-(OH) 2D3] or all-trans retinoic acid (all-trans RA) (Kim *et al.*, 2003). This study showed by using 10μM of artemisinin alone, artemisinin presented weak effects in terms of differentiation in HL-60 cells where ERK inhibitors were shown to block this effect.

The artemisinin's are currently undergoing widespread investigation as potential chemotherapeutic agents to be used in the treatment of a variety of cancers. They are known to generally inhibit tumour growth, induce growth cycle arrest, promote apoptosis, negate angiogenesis and prevent tissue invasion and metastasis (Crespo-Ortiz and Wei, 2012; Ho *et al*., 2014). Our results show artemisinin exerts additive or synergistic effects on HL-60 viability which agrees with previous research.

In contrast to presently prescribed anthracyclines, clinical toxicology studies involving malaria patients, where the artemisinins have been extensively researched, have shown minimal side effects and resistance has been observed, although some presented slight

neurotoxicity (Efferth & Kaina, 2010; Liang and Albrecht, 2003; Dondorp *et al*., 2010; McGready *et al*., 2012).

Clinical trials have reported that use of the iron-chelating *dexrazoxane*, a cardioprotective agent, will effectively prevent the cumulative anthracyline induced cardiotoxicity from doxorubicin and epirubicin treatment in patients with metastatic or advanced breast cancer (Minotti *et al.*, 2001). Minotti *et al.* (2001) have documented that changes in iron homeostasis are major players in cardiotoxicity induced by doxorubicin, leading to the generation of by-products such as the secondary alcohol doxorubicinol or ROS, which act independently or as part of the same mechanism. Doxorubicinol and ROS have both been shown to contribute to cardiotoxicity by inactivating iron regulatory proteins (IRP) and altering the function of cytoplasmic aconitase/IRP-1, leading to the accumulation of excess iron within the cells. Excess iron undoubtedly causes a cluster formation of IRP-1 in cells, which generates null proteins that prevents miRNA binding, leading to a switch to aconitase. This prevents iron uptake for normal metabolic use and also prevents iron homeostasis, which induces the severe cardiotoxicity observed in treatment (Minotti *et al*., 2001).

For this reason, dexrazoxane, despite being a cardioprotective agent, is now contraindicated in children and young adolescents, as it causes severe myelosuppression and serious infection (Schwartz *et al*., 2009). Its use has been limited to adults with advanced or metastatic breast cancer who have previously received a minimum cumulative dose of 300 mg/m^2 doxorubicin or 540 mg/m^2 epirubicin (Schwartz *et al.*, 2009; Swain *et al.*, 1997). As such, deferasirox is unable to provide cardioprotection against anthracycline-induced cardiotoxicity (Simunek *et al.*, 2009). Although several agents have been identified to be cardioprotective against doxorubicin-induced cardiotoxicity, such as the beta-blocker carvedilol (Carreira *et al.*, 2006), curcumin (Srivastava and Mehta, 2009), flavonoids (Bast *et al*., 2007), erythropoietin (Kim *et al.*, 2008), antioxidants (Koti *et al*., 2008) and many others, the molecular pathogenesis of anthracycline cardiotoxicity remains highly controversial, making the possible effectiveness of the anthracycline cardioprotective agent complex highly debatable depending on the nature of the compound. Many anthracyclines may promote the formation of ROS through redox cycling of their aglycones. These free radicals can also cause lipid peroxidation which can lead to cell damage (Banfi *et al.*, 1992).

Doxorubicin induced ROS which is found to activate cell death pathways which explains doxorubicin induced cardiotoxicity in cardiomyocytes (Montaigne *et al.*, 2012; Sawyer *et al.*, 2010). Elbaky *et al.* (2010) co-administered simvastatin with doxorubicin in a cardiovascular rat model and demonstrated that simvastatin protects against myocardial injury and decreases doxorubicin induced stress. With decades of research, artemisinin has shown much promise, with reports showing very minimal or no side effects with its clinical use, especially in the malaria setting, where there are tight measures to contain artemisinin resistance in malaria endemic areas (Nakase *et al*., 2008; WH0 2011). Therefore, there is a great potential in developing arteminin as a cardioprotective agent against doxorubicin induced cardiotoxicity. There is also evidence that the antitumour effect of certain cancer chemotherapy agents may be augmented by the presence of nitric oxide (Wink *et al.*, 1991). Nitric oxide can therefore enhance the effect of doxorubicin, and with artemisinin shown in chapter 5 to generate nitric oxide via eNOS. The synergistic anticancer activity may be enhanced by using the combination of artemisinin and doxorubicin in cancer treatment.

Based on the results from the studies above, we have successfully established that by using the inhibitors of nitric oxides (L-NAME and aminoguanidine), we can now suggest that artemisinin's reversal of doxorubicin induced cardiotoxicity in the H/R setting may be associated with the nitric oxide cell survival siganalling pathway. This pathway is a known cardioprotective pathway (Heusch *et al*., 2008; Wang *et al*., 2008) and one that can be attributed to artemisinin's cardioprotection in our previous chapter. This finding allows us to implicate nitric oxide involvement in cardioprotection. Previous studies have howerever established in HL-60 cells the involvement of ERK in their differentiation to granulocytic and monocytic lineage known as MAPK in HL-60 and NB4 cells (Kim *et al.*, 2003)

Furthermore findings from this study have suggested a dual role for artemisinin in cardiomyocytes as well as in HL-60 cell, both involving nitric oxide pathway. However, further studies could investigate the effect of iNOS and eNOS expression on HL-60 cells using FACS analysis. The study proposes artemisinin has powerful cardioprotective effect against I/R injury and doxorubicin induced cardiomyopathy in a variety of settings, as well as artemisinin being a potent cytotoxic agent against HL-60 cells with both effects

implicating the nitric oxide synthase pathway. Previous studies have implicated nitric oxide in mediating anti-hypertrophic effects, with artemisinin showing anti-hypertrophy in a dose-dependent manner in cardiomyocytes (Wollert *et al.*, 2002; Xiong *et al.*, 2010). Kukreja (2007) similarly showed using a cardioprotective agent against I/R, doxorubicin induced cardiomyopathy and anti-hypertensive effect was induced by the chronic nitric oxide synthase pathway in animals which agrees with our findings in isolated perfused hearts and cardiomyocytes.

We can therefore suggest artemisinin's profound anti-cancer activity to be via the mediation of nitric oxide in HL-60 cells. This study showed promising cytotoxicity using artemisinin treatment against HL-60 cells. This is supported by research by Aldieri *et al*. (2003) who showed artemisinin inhibited nitric oxide synthesis in cytokine-stimulated human astrocytoma T67 cells and activated the nuclear factor NF-kB (Aldieri *et al*., 2003). A study has suggested that artemisinin is responsible for the cytotoxic effects in HL-60 cells by the release of the highly alkylating carbon-centered radicals and ROS (Uysal, Sahna, Ozguler, Burma, & Ilhan, 2014) which causes cellular necrosis in HL-60 cells. Another study suggested artemisinin is a possible treatment in leukaemic disease as shown by their promising results (Kim *et al.*, 2003). Tumour cells are more prone to ROS damage as they exhibit very low expression of antioxidant enzymes such as superoxide dismutase, glutathione peroxidase, catalase as compared to the function and damage done in normal cells. Hence, increasing oxidative stress to allow an antitumor mechanism to become active is common in the anticancer medication procedures. Furthermore we have shown upon addition of doxorubicin, artemisinin's mediated protection against I/R and doxorubicin induced injury as well as its combined cytotoxicity implicate the nitric oxide pathway as summarised in the schematic representation in Figure 61.

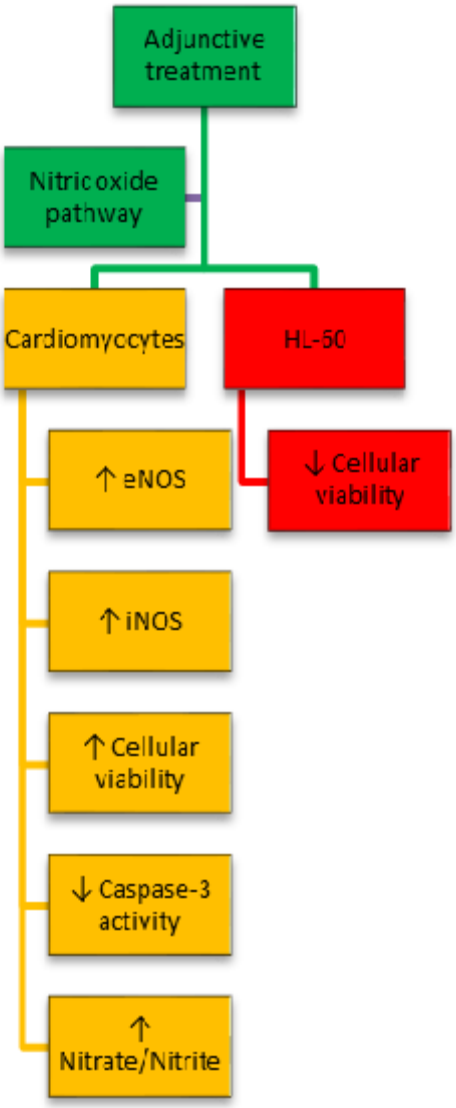


Figure 61: Hypothetical schematic representation of the adjunctive therapy using artemisinin and doxorubicin in isolated cardiomyocytes and isolated perfused heart subjected to HR injury and I/R respectively via the downstream targets of PI3K-Akt kinase cascade, Nitric oxide. Administering Artemisinin (4.3µM)and doxorubicin (1µM) at reperfusion initiated cardioprotection by protecting against reperfusion-induced cell death in cardiomyocytes. The scheme portrays the important anti-apoptotic mechanisms that have been implicated in mediating cellular survival associated with the recruitment of nitric oxide shown to reverse the activation of caspases and increase nitrite and nitrate production. Doxorubicin (1µM) and artemisinin (0.4mM) nearly doubled doxorubicin cytotoxicity in HL-60 cell line.

6.5 CONCLUSION

We have successfully shown, by co-administering artemisinin and doxorubicin at reperfusion, that artemisinin attenuates and protects against both I/R injury and doxorubicin-induced injury in isolated perfused rat hearts in a nitric oxide dependent manner. Co-treating the isolated cardiomyocytes with artemisinin and doxorubicin reversed the H/R and doxorubicin induced injury decreased the levels of cleaved caspase 3 compared to the H/R control group. Artemisinin was shown to confer protection via activation of nitric oxide synthase pathways in both the I/R setting as well as in H/R setting shown to upregulates p-eNOS protein significantly compared with time matched controls. Nitric oxide was also generated via iNOS expression with artemisinin treatment.

Additionallly, this study investigated the effect of artemisinin alone in HL-60 cancer cells and upon co-treating with doxorubicin in HL-60 cells and in cardiomyocytes. Artemisinin was shown to confer protection in the isolated perfused heart model and in isolated cardiomyocytes subjected to H/R injury and doxorubicin induced injury. Cleaved caspase-3 levels were shown to decrease and cellular viability to improve. Co-treatment with L-NAME and aminoguanidine implicated the nitric oxide pathway in cardioprotection. In HL-60 cells, we also achieved treatment efficacy in terms of cytotoxicity using artemisinin as an adjuvant to doxorubicin without compromising on doxorubicin's effectiveness against HL-60 cells. In the process, we established that artemisinin and doxorubicin administered together present an enhanced cytotoxicity in a nitric oxide dependent manner. We also found that by administering the two together doubled artemisinins cytotoxicity while implicating the nitric oxide pathway in HL-60 cells.

The result from this study demonstrated the value of adjuvant treatment and identified that artemisinin augments the cytotoxicity of doxorubicin in HL-60 cells. The study also provided valuable information about the cell signalling pathway involved in co-administering artemisinin and doxorubicin in cardiomyocytes and in HL-60 cells which is suggested to occur via a nitric oxide dependent manner. The use of artemisinin as an adjuvant regimen to doxorubicin treatment although warrants further investigation shows a new opportunity to enhance the effectiveness of doxorubicin treatment in cancer treatment.

Chapter 7

7 GENERAL DISCUSSION

7.1 DISCUSSION

Summary of main findings

In this study we have shown that:

- Treatment with artemisinin resulted in a decrease myocardial infarction, increase in cellular viability, decrease in levels of cleaved caspase-3 in isolated perfused hearts and isolated cardiomyocytes subjected to I/R and H/R respectively.
- Hearts treated with artemisinin showed a decrease in the expression of miRNA associated with apoptosis and myocardial injury.
- Artemisinin limits myocardial reperfusion injury via the recruitment of PI3K-Akt-p70s6k/BAD cell survival pathway which may offer a novel strategy for cardioprotection in a clinical setting.
- Artemisinin treatment led to an increase in the levels of iNOS and eNOS in isolated cardiomyocytes. This study implied the presence of eNOS may be associated with artemisinin's protective effect against myocardial reperfusion injury. It furthermore associated the treatment with increase in nitrate and nitrite in a dose dependent manner thus implying the role of nitric oxide in the observed protection.
- Artemisinin was shown to have profound anticancer activity against HL-60 cells.

- Co-treating artemisinin with doxorubicin showed artemisinin confers protection against I/R injury and doxorubicin induced injury in isolated perfused hearts in a nitric oxide dependent manner.
- Combined treatment of artemisinin and doxorubicin showed a decrease in cleaved caspase 3 levels, improvement in cardiomyocytes viability and enhanced cytotoxic efficacy against HL-60 cells. Furthermore establishing the enhanced cytotoxicity is associated with nitric oxide pathway.
- The findings generally suggest artemisinin is pro-apoptotic in HL-60 cells and anti-apoptotic in the myocardium which suggests the potential of artemisinin particularly in cancer patients with underlying comorbities such as IHD.
- Findings also suggest artemisinin to be a promising remedy in combating a variety of diseases beyond malaria.

Previous research has shown no evidence of cardiotoxicity with artemisinin or its derivatives when administered alone in antimalarial patients (Vugt *et al*., 1996; Gupta *et al.*, 2005). However, a controversial study recently published that by administering artesunate-amodiaquine in the treatment of uncomplicated malaria, in a study involving Ghanian children, patients presented incidences of sinus bradycardia (Adjei *et al.,* 2012). This was however concluded that the rhythmic disturbances may well be associated with amodiaquine rather than artesunate (Adjei *et al.,* 2012).

Several studies have confirmed artemisinin, which contains an endoperoxide bridge is the structure responsible for its mechanism of action in antimalarial treatment (Posner & O'Neill, 2004). As well as being used as an antimalarial drug, artemisinin has a great potential in the treatment of several cancer types (Efferth *et al.,* 2011). However in this thesis we focused on the effect of artemisinin in the myocardium, a popular off-target of drug toxicity.

As described in previous chapters, upon reperfusing an occluded organ (after a transient period of ischaemia), reperfusion injury often occurs. This is described as one of the major

causes of damage to tissues (Uysal *et al.*, 2014). During ischaemia, hearts also undergo inflammation of vascular tissues, oxidative damage, cellular apoptosis and necrosis leading to ischaemia reperfusion injury (Wang *et al.*, 2007). There is a lot of recent developments in the field of oxidative stress research, highlighting the role of ROS, inflammation and I/R injury. Toxic oxidative reaction such as lipid peroxidation, inhibition of mitochondrial respiratory chain enzymes, inactivation of membrane sodium channels, and other oxidative modifications of proteins have been related to named pathophysiologies. Several novel findings have identified novel pharmacological agents such as peroxynitrite decomposition catalysts and selective superoxide dismutase mimetics (SODm) in shock, I/R, and inflammation. Treatment with peroxynitrite decomposition catalysts inhibits peroxynitrate while SODm selectively mimics the activity of the human superoxide dismutase enzymes. *In vivo* studies have been shown that this treatment helps to cellular energetic failure associated with shock, inflammation, and I/R injury. ROS has also previously been associated with initiating DNA single-strand breakage and activation of the nuclear enzyme poly (ADP-ribose) synthetase thus leading to severe energy depletion of the cells and necrotictype cell death. Treatment with antioxidant has been shown to inhibit the activation of poly(ADP-ribose) synthetase thus preventing organ injury associated with shock, inflammation, and I/R (Cuzzocrea *et al.*, 2001). Studies like ours also show the potential of artemisinin as a pharmacological agent capable of salvaging cardiomyocytes subjected to I/R and H/R injury. The study ahs identified potenatial mechanisms of artemisinin mediated cardioprotection which will undoubtedly help in developing artemisinin as a potential treatment against I/R injury.

This project investigated the effects of artemisinin treatment in stressed conditions. It also looked at the intracellular mechanism associated with the observed cardioprotective effect. Previous study by Gu et al. (2012) which supports the cardioprotective properties of artemsinin we have similar shown suggests that the protection observed is via the down regulation of NFkB pathway although the study failed to investigate the effect of artemisinin treated sham group which would have clearly elucidated the effects of artemisinin treatment thus making their results more convincing. Furthermore, studies by Purcell et al. (2001) and Gupta et al. (2002) suggest that the activation of the NFkB pathway is usually a causal effect in cardiac hypertrophy response (Gu *et al.*, 2012).

Several assays using cardiomyocytes were used for the screening of artemisinin in order to determine its mechanistic basis in cardiomyocytes such as MTT assay, cleaved-caspase-3, nitric oxide assay and flow cytometric analysis. The effects on cell proliferation (in HL-60 cells) was also investigated using MTT assay to investigate artemisinin's direct cytotoxic effects against HL-60. Understanding of the intracellular signalling involved in artemisinin mediated cardioprotection will provide an insight and inform future development of non toxic cancer therapies. This project also, investigated the differential expression of some popular miRNA's associated with I/R. We furthermore investigated the potential of administering artemisinin as an adjunct therapy to the cardiotoxic doxorubicin in isolated cardiomyocytes and HL-60 cancer cells.

Our data showed a dose dependent decrease in infarct size which is consistent with Sun *et al*., (2007) who showed artemisinin at 10µM and 100µM alleviated myocardial injury in experimental Wistar rats which they believe may be associated with its functions of anti-oxidation and scavenging of free radicals. The ability of artemisinin to alleviate the simulated I/R injury by attenuating and preventing cellular damage proves artemisinin is an agent with great potential. However, we also showed artemisinin improves myocytes viability as well as offers protection via an anti-apoptotic process. This study, further investigated the intracellular signalling pathways involved in this observed protection by co-administering inhibitors of PI3K pathway (wortmannin) and inhibitor of p70S6K (rapacymin) where both inhibitors abolished the observed protection thus suggesting the involvement of the PI3K-Akt-BAD/p70s6K pathway. Studies have previously shown, Akt in the mitochondria translocates to sites where it modulates signalling for either cell death or cell survival (Yang *et al*., 2009 and Abel and Doenst 2011).

Our results show a significant increase in the level phosphorylated Akt with artemisinin treatment and a subsequent decrease was observed by co-administering artemisinin with the inhibitor of PI3k-Akt, wortmannin. This suggests the involvement of PI3k-Akt pathway of mediation in artemisinin's cardioprotection. We also investigated the downstream targets of Akt such as BAD and p70S6K.

Using Western blotting and flow cytometric analysis, we confirmed that artemisinin protected the isolated perfused heart and the ventricular myocytes from the ischaemia reperfusion injury via the recruitment of PI3K-Akt-BAD/p70s6K cell survival pathway.

It is well established in research that the activation of PI3K-Akt-BAD/p70s6K cell survival pathway is associated with the activation of other pro-survival factors (such as G-protein-coupled receptor ligands, bradykinin) and inhibition of pro-apoptotic factors such as BAX, p53 which can be investigated in further studies (Li and Sato, 2001; Al khouri *et al.*, 2005; Hausenloy and Yellon, 2004). Hausenloy and Yellon (2004) also showed signalling through PI3K-Akt-BAD pathway confers protection against I/R injury, through the activation of the serine–threonine kinase, Akt (Hausenloy and Yellon 2004) thus supporting our findings. Studies have confirmed the association of PI3K-Akt-p70s6K in cardioprotection in the past, in studies using pharmacological agents such as cordyceps which similarly has an anticancer, anti-inflammatory, and antioxidant function and was shown to alleviate myocardial I/R injury in Sprague Dawley rats (Park *et al.*, 2014). The PI3K-Akt-p70s6K is well established as a cardioprotective pathway as previously described.

We also investigated another downstream target of PI3K pathway, nitric oxide and found artemisinin attenuates I/R injury via the upregulation of eNOS. There is overwhelming evidence that shows nitric oxide is an important cardioprotective molecule due to its vasodilator, antioxidant, antiplatelet, and anti-neutrophil actions making it essential for normal cellular homeostasis (Ferdinandy 2006).

Myocardial function remained relatively stable in both our ischaemic and normoxic model. The IC_{50} used in a previous study by Hara *et al*., (2007) which was 30µM (nearly 15 times higher than the normal plasma concentration used in a clinical setting for antimalarial treatment which is 2.1µM after an oral administration of 500mg) (Alin *et al*.,1996) expressed anticholinergic toxicities. The possible anti cholinergic toxicities that maybe observed with the use of artemisinin may only be evident at a concentration 15 times the plasma concentration presently administered according to the aforementioned study. This further suggests artemisinin at lower concentrations and at clinical concentrations will not

influence heart function. Other studies have also verified the influence of artemisinin in the heart may only appear at doses higher than normal plasma concentrations.

Artemisinin in the heart counteracts the apoptotic effect induced by H/R injury enabling less myocytes to be committed to apoptosis. Our results implied the process is mediated by the joint effect of the phosphorylation of Akt in the cardiac cells as well as the suppression of caspase-3 activation. Experimental studies have shown that by administering cardioprotective agents such as erythropoietin (shown to be anti-apoptotic), PI3K-γ/δ inhibitors (shown to be anti-inflammatory), intracoronary aqueous oxygen, and ischaemic post condiitoning (shown to be anti-apoptotic and anti-inflammatory) have shown promising results of limiting acute myocardial infarction. An acute therapeutic window is also reported to exist in patients with STEMI whereby administering a pharmacological intervention few hours after PPCI procedure might provide an additional therapeutic window to target late into the reperfusion phase (Hausenloy and Yellon, 2013). This may be a great investigational area of interest whereby artemisinin can be studies and developed as a novel treatment to limit myorcardial injury.

Cleaved caspase-3, is also a pivotal effector caspase in apoptotic signalling which has been associated with I/R and cellular damage leading to apoptosis (Sakamaki and Satou 2009; Lu and Chen 2011). Artemisinin administered during re-oxygenation also showed improvement in the viability of isolated ventricular myocytes and it was able to decrease a cleaved caspase-3 activity in the isolated perfused heart. This is indicative of the potential of artemisinin to salvage H/R induced injured adult ventricular myocytes and suggest the action artemisinin's action is via anti-apoptotic mechanisms. Artemisinin in cancer cells also creates free radicals that are carbon-centered and this may facilitate lysosomal disturbance and generate ROS which results in mitochondrial impairment, activation of caspases, and cell death (Wang *et al.*, 2007). In the mitochondrion, these adducts interfere with the electron transfer chain (ETC) by interacting with heme or heme-bound proteins leading to generation of ROS and apoptosis (Uysal *et al*., 2014).

A recent study has confirmed the generation of ROS in artesunate-treated HL-60 cells after 16hrs of treatment suggesting the mechanism of artemisinin induced damage in cancer cells

(Uysal *et al.*, 2014). This study also showed administering artemisinin reverses the doxorubicin induced toxicity by decreasing the infarct sizes in isolated perfused rat hearts subjected to I/R injury and in cardiomyocytes.

In the MTT assay using HL-60 cancer cells, artemisinin expressed profound cytotoxicity due to its ability to react with iron in cancer cells to form free radicals (Lai *et al.*, 2005). In most of the systems, artemisinin's cytotoxicity is often aggravated by the cancer cells that have an exaggerated iron expression which increases artemisinin's activity around 100-fold compared to other normal cell lines, as we observed in our artemisinin treated HL-60 cancer cells compared to control (Posner and O'Neill, 2004). Cancer cells exhibit a rise in transferrin receptors (TfR) which are responsible for intracellular concentration levels and the uptake in iron levels in the system. The different levels of TfR in the cancer cells may be variable depending on the cell line (Lai *et al.*, 2013). A theory suggested that iron-activated artemisinin is responsible for the damage caused by the release of the highly alkylating carbon-centered radicals and ROS (Uysal *et al.*, 2014). Tumor cells are more prone to ROS damage as they exhibit very low expression of antioxidant enzymes such as superoxide dismutase, glutathione peroxidase, catalase as compared to the function and damage done in normal cells (Uysal *et al.*, 2014).

The drug concentrations which are required to produce damage in cancer cells are often higher than the amounts required to induce toxicity in malaria parasites. As we have shown in our study also the concentration required to be protective in cardiomyocytes is 100 fold less than in cancer cells. Artemisinin, artemether, DHA and artesunate exhibit IC_{50}s (fifty percent inhibitory concentration) up to 15 nM in malaria parasites. Microscopy analyses in artesunate-treated cells have displayed early oncosis-like morphological changes which activate the ROS generation in the subcellular structures (Uysal *et al.*, 2014).

In spite of the growing indication of the damage affected by ROS system in many cells, cell damage is also related with oxidative stress. The novel artemisinin dimers are able to incur damage in the cancer cells without being dependent on the generation of ROS, however, the underlying mechanism of cytotoxicity is another matter. It is also not proven whether

artemisinin's-induced cell death may or may not be a ROS-independent mechanism of the death of cancer cells.

Doxorubicin was shown to cause cardiac injury and increased the infarct size in both during ischaemia/reperfusion injury and in naive conditions. Studies have also associated doxorubicin with adverse cardiac effects, including progressive heart failure and cardiomyopathy (Gharanei *et al.*, 2013). Artemisinin was used as an adjunctive therapeutic agent which reverses doxorubicin induced injury in ischaemia reperfusion setting. In addition to this findings, our study showed by administering the adjunctive treatment, data showed a profound anti-cancer effect (nearly doubling their individual cytotoxic effect). Results also showed this effect is in a nitric oxide dependent manner. Previous studies have implicated Nitric oxide in preventing cancer metastasis and inhibition of cell adhesion molecules (CAMs) (Efferth *et al.*, 2011).

In our miRNA study, we investigated the miRNA expression changes in response to treatment with artemisinin. Despite artemisinin showing protection in animal studies, artemisinin needs to be fully studied in clinical trial setting and the development of miRNAs as reliable biomarkers for early assessment of potential drug induced cardiotoxicity especially in patients with comorbidities or at risk of developing cardiac myopathies is highly important. As this could allow the detection of subclinical cardiac injury (Sandhu and Maddock 2014).

Furthermore, this study showed that artemisinin alleviated I/R injury and doxorubicin induced cardiotoxicity in isolated perfused hearts, doxorubicin decreased haemodynamic functions and increased infarct to risk ratio in I/R tissue which artemisinin was shown to reverse. This study has also showed that artemisinin has a dose dependent decrease in I/R and improves viability in the MTT assay shown as an increase in nitric oxide synthesis via p-eNOS and iNOS synthesis with adjunctive treatment. High levels of nitric oxide produced via eNOS synthesis are well established as cardioprotective where studies in the past have implicated the PI3K-eNOS pathway in protection as show in our study (Bell and Yellon 2002). Other studies have also shown low levels of nitric oxide generated via iNOS

synthesis may present cardioprotection (Cole *et al.*, 2006). Both studies although contrasting in view support our findings.

7.2 STUDY LIMITATIONS/FUTHER SUGGESTIONS

In the current study we observed some effects on haemodynamic function of the heart following artemisinin and doxorubicin administration. However, it will be worth investigating the effect of reduction in function. The langendorff model itself exposes the excised heart to several vulnerabilities such as injury due to instrumentation although accounted for by the control hearts which could also increase the possibility of preconditioning due to cannulation of the heart. Research has shown that generation of free radicals in ischaemic heart diseases have a akinetic effect on the heart muscle, there is a limitation and discrepancy in findings in a controlled environment (laboratory) and that found in clinical trials and upon administration in clinics.

Speculations have also been made about reaching a therapeutic ceiling with current reperfusion treatment. Reperfusion has been shown to limit infarct size by approximately 50%, in a review by Miura and Miki (2008) using a consensus of over 10 studies reported patients showed more than 75% infarction despite coronary reperfusion suggesting mortality and morbidity is increased when more than 20% of the left ventricle is infarcted thus suggesting effective reperfusion treatment has to reduce infarction to below the 20% target (Miura and Miki 2008). However, with research by Hausenloy and colleagues which suggested ,a mediator pathway that is activated in reperfusion, have revealed in their study that cardioprotection occur as a result of remote preconditioning by inhibiting transition pores at reperfusion however, half of the dead cells were killed by transition pores in early reperfusion (Zhoa *et al.,* 2003). Many strategies for activating preconditioning have been developed based on this and using several off patent compounds such as GSK blockers, erythropoietin, cyclosporine A, nitric oxide donors and so on. This in my opinion is because there is very little interest in cardioprotectants by the big pharmaceutical companies due to several failed trials and too many off patent compounds leaving the larger trails to be government sponsored such as the cyclosporine A trail by Ovize et al (2011). In a review by King (2010), he reported Ovize *et al* (2011) had shown that infarct size in patient undergoing

reperfusion therapy alone was strongly correlated with risk zone which we evaluated in our study. Identifying the risk can easily identify the 25% that needs treatment (Piot *et al*., 2008). Animal models respond well to preconditioning based interventions but whether the lack of a clear end point as shown in experimental studies will limit the development remains a concern (Piot *et al.*, 2008).

Large scale clinical trials such as the AMISTAD I have produced positive results via subgroup analysis whereas applying the same analysis to a larger trails AMISTAD II failed to show similar results (Ross *et al.*, 2005) this raises concerns of reproducibility and clinical trials often not as robust as experimental studies those questioning the suitability in clinical appications.

UO126 should be used in further study to identify whether the increase in the p42/p44 extra-cellular signal-regulated kinases (ERK 1/2) protein levels thus investigating both branches of the RISK pathway for possible association. ERK 1/2 is also known to confer powerful cardioprotective responses. These two pathways (PI3K-Akt and Erk 1/2) can however either work together or independently and at the moment is outside the scope of our research.

Myocardial I/R injury is undoubtedly a multifactorial phenomenon resulting in damage inflicted on the myocardium. This is shown to range from atherosclerotic plaque rupturing, formation of plugins in the microcirculation upon restoring blood flow across the obstructed site, neutrophil aggregation, tissue oedema and more. The therapies being used include mechanical opening of the vessel followed by pharmacological intervention such as adjuvant use of anti coagulants in reducing ischaemic injury and therapies aimed at limiting the extent of necrosis have proven quite successful in limiting ischeamic injury (Ibáñez *et al.*, 2015). The time has come to focus on therapies to reduce reperfusion injury, few studies have been relatively successful, we proposed the use of pharmacological agents such as artemisinin shown to have multiple effects such as anti inflammatory effects in addition to the previously discussed might be a viable way of reducing infiltration of myocardial tissue by inflammatory cells which is known to induce an additional damage to the myocardium. However, until sufficiently reviewed, artemisinin remains a potential therapy requiring development in the filed of I/R.

Although no unfavourable effects were observed with artemisinin treatment, additional experiments with various treatment timings are desirable. The effect of continuous administration is similarly as important as using different dosages as we did in the present study. In order to increase the impact of this study, using an aged population where comorbidities are more likely to occur will significantly enhance the impact.

Furthermore, artemisinin in malarial treatment has been shown to involve the parasites mitochondria where it causing dysfunction and eventually death due to mitochondrial mediation. Investigating mPTP in cardiomyocytes woud have provided a better insight into our study as they may be changes in the respiratory chain depolarization of the mitochondrial membrane or from the prevention of ROS generation and calcium uptake in cells however investigating nitric oxide via mPTP is problematic due to the ubiquitous role of nitric oxide and the fact that the mitochondria appear to be a target for nitric oxide-mediated injury (Borutaite *et al*., 2000).

7.3 CONCLUSION

This study demonstrated the protective effects of artemisinin in stressed conditions such as I/R and/or doxorubicin induced injuries. To our knowledge this is the first study to elucidate the intracellular signalling involved in artemisinin's cardioprotectiveness involves PI3K-AKT-BAD/p70S6K in an ex vivo model of the myocardium subjected to I/R injury as well as the first study to implicate the generation of nitric oxide via eNOS and iNOS synthesis in artemisinin in cardiomyocytes.

It also demonstrated that artemisinin can not only reverse I/R injury but also doxorubicin induced toxicity in isolated perfused hearts and isolated cardiomyocytes subjected to HR injury. Co-administration of artemisinin and doxorubicin also more than double the anti-cancer effect of both drugs HL-60 cells.

8 REFERENCES

Abel, E. D. And Doesnt, T. 2011. Mitochondrial adaptations to physiological vs. pathological cardiac hypertrophy. *Cardiovasc Res,* 90**,** 234-42.

Abraham, R. T. (2001) 'Cell Cycle Checkpoint Signaling through the ATM and ATR Kinases'. *Genes & Development* 15 (17), 2177-2196

Abu-Soud, H.M., Ichimori, K., Presta, A and Stuehr D.J. (2000) 'Electron Transfer, Oxygen Binding, and Nitric Oxide Feedback Inhibition in Endothelial Nitric-oxide Synthase'. *Journal of Biological Chemistry*. 275, 17349-17357

Achan, J., Talisuna, A. O., Erhart, A., Yeka, A., Tibenderana, J. K., Baliraine, F. N., Rosenthal, P. J., and D'Alessandro, U. (2011) 'Quinine, an Old Anti-Malarial Drug in a Modern World: Role in the Treatment of Malaria'. *Malaria Journal* 10, 144-2875-10-144

Adams, M. J. and Lipshultz, S. E. (2005) 'Pathophysiology of Anthracycline- and Radiation-Associated Cardiomyopathies: Implications for Screening and Prevention'. *Pediatric Blood & Cancer* 44 (7), 600-606

Adao, R., de Keulenaer, G., Leite-Moreira, A., and Bras-Silva, C. (2013) 'Cardiotoxicity Associated with Cancer Therapy: Pathophysiology and Prevention Strategies'. *Revista Portuguesa De Cardiologia: Orgao Oficial Da Sociedade Portuguesa De Cardiologia = Portuguese Journal of Cardiology: An Official Journal of the Portuguese Society of Cardiology* 32 (5), 395-409

Agarwal, N. K., Qu, C., Kunkalla, K., Liu, Y., and Vega, F. (2013) 'Transcriptional Regulation of Serine/Threonine Protein Kinase (AKT) Genes by Glioma-Associated Oncogene Homolog 1'. *Journal of Biological Chemistry*

Aghaeepour, N., Finak, G., FlowCAP Consortium, DREAM Consortium, Hoos, H., Mosmann, T. R., Brinkman, R., Gottardo, R., and Scheuermann, R. H. (2013) 'Critical Assessment of Automated Flow Cytometry Data Analysis Techniques'. *Nature Methods* 10 (3), 228-238

AHA (2014) ‘Heart attack or sudden cardiac arrest: How are they different?’ Available online from< http://www.heart.org/HEARTORG/Conditions/More/MyHeartandStrokeNews/Heart-Attack-or-Sudden-Cardiac-Arrest-How-Are-They-Different_UCM_440804_Article.jsp>

Ai, J., Zhang, R., Li, Y., Pu, J., Lu, Y., Jiao, J., Li, K., Yu, B., Li, Z., Wang, R., Wang, L., Li, Q., Wang, N., Shan, H., Li, Z., and Yang, B. (2010) 'Circulating microRNA-1 as a Potential Novel Biomarker for Acute Myocardial Infarction'. *Biochemical and Biophysical Research Communications* 391 (1), 73-77

Akter, R., Hossain, M. Z., Kleve, M. G., and Gealt, M. A. (2012) 'Wortmannin Induces MCF-7 Breast Cancer Cell Death Via the Apoptotic Pathway, Involving Chromatin Condensation, Generation of Reactive Oxygen Species, and Membrane Blebbing'. *Breast Cancer (Dove Medical Press)* 4, 103-113

Allen, D. G., Lamb, G. D., and Westerblad, H. (2008) 'Skeletal Muscle Fatigue: Cellular Mechanisms'. *Physiological Reviews* 88 (1), 287-332

Aldieri, E., Atragene, D., Bergandi, L., Riganti, C., Costamagna, C., Bosia, A., and Ghigo, D. (2003) 'Artemisinin Inhibits Inducible Nitric Oxide Synthase and Nuclear Factor NF-kB Activation'. *FEBS Letters* 552 (2-3), 141-144

Alin, M. H., Ashton, M., Kihamia, C. M., Mtey, G. J., and Bjorkman, A. (1996) 'Clinical Efficacy and Pharmacokinetics of Artemisinin Monotherapy and in Combination with Mefloquine in Patients with Falciparum Malaria'. *British Journal of Clinical Pharmacology* 41 (6), 587-592

Al-Khouri, A. M., Ma, Y., Togo, S. H., Williams, S., and Mustelin, T. (2005) 'Cooperative Phosphorylation of the Tumor Suppressor Phosphatase and Tensin Homologue (PTEN) by Casein Kinases and Glycogen Synthase Kinase 3beta'. *The Journal of Biological Chemistry* 280 (42), 35195-35202

Álvarez, P., Tapia, L., Mardones, L. A., Pedemonte, J. C., Farías, J. G., and Castillo, R. L. (2014) 'Cellular Mechanisms Against Ischaemia Reperfusion Injury Induced by the use of Anesthetic Pharmacological Agents'. *Chemico-Biological Interactions* 218 (0), 89-98

Anderson, J. L., Carlquist, J. F., Horne, B. D., and Hopkins, P. N. (2007) 'Progress in Unraveling the Genetics of Coronary Artery Disease and Myocardial Infarction'. *Current Atherosclerosis Reports* 9 (3), 179-186

Annapurna, A., Ansari, M. A., and Manjunath, P. M. (2013) 'Partial Role of Multiple Pathways in Infarct Size Limiting Effect of Quercetin and Rutin Against Cerebral Ischaemia-Reperfusion Injury in Rats'. *European Review for Medical and Pharmacological Sciences* 17 (4), 491-500

Apostolova, N. and Victor, V. M. (2015) 'Molecular Strategies for Targeting Antioxidants to Mitochondria: Therapeutic Implications'. *Antioxidants & Redox Signaling* 22 (8), 686-729

Apraiz, A., Boyano, M. D., and Asumendi, A. (2011) 'Cell-Centric View of Apoptosis and Apoptotic Cell Death-Inducing Antitumoral Strategies'. *Cancers* 3 (1), 1042-1080

Araneda, O. F. and Tuesta, M. (2012) 'Lung Oxidative Damage by Hypoxia'. *Oxidative Medicine and Cellular Longevity* 2012, 856918

Araújo, N. C. P., Barton, V., Jones, M., Stocks, P. A., Ward, S. A., Davies, J., Bray, P. G., Shone, A. E., Cristiano, M. L. S., and O'Neill, P. M. (2009) 'Semi-Synthetic and Synthetic 1,2,4-Trioxaquines and 1,2,4-Trioxolaquines: Synthesis, Preliminary SAR and Comparison with Acridine Endoperoxide Conjugates'. *Bioorganic & Medicinal Chemistry Letters* 19 (7), 2038-2043

Armand, P. (2015) 'Immune Checkpoint Blockade in Hematologic Malignancies'. *Blood* 125 (22), 3393-3400

Arsenault, P. R., Wobbe, K. K., and Weathers, P. J. (2008) 'Recent Advances in Artemisinin Production through Heterologous Expression'. *Current Medicinal Chemistry* 15 (27), 2886-2896

Asano, G., Takashi, E., Ishiwata, T., Onda, M., Yokoyama, M., Naito, Z., Ashraf, M., and Sugisaki, Y. (2003) 'Pathogenesis and Protection of Ischaemia and Reperfusion Injury in Myocardium'. *Journal of Nihon Medical School = Nihon Ika Daigaku Zasshi* 70 (5), 384-392

Asnaghi, L., Calastretti, A., Bevilacqua, A., D'Agnano, I., Gatti, G., Canti, G., Delia, D., Capaccioli, S., and Nicolin, A. (2004) 'Bcl-2 Phosphorylation and Apoptosis Activated by Damaged Microtubules Require mTOR and are Regulated by Akt'. *Oncogene* 23 (34), 5781-5791

Azizzadeh, B., Yip, H. T., Blackwell, K. E., Horvath, S., Calcaterra, T. C., Buga, G. M., Ignarro, L. J., and Wang, M. B. (2001) 'Nitric Oxide Improves Cisplatin Cytotoxicity in Head and Neck Squamous Cell Carcinoma'. *The Laryngoscope* 111 (11 Pt 1), 1896-1900

Baehrecke, E. H. (2005) 'Autophagy: Dual Roles in Life and Death?'. *Nature Reviews.Molecular Cell Biology* 6 (6), 505-510

Baines, C. P. (2011) 'How and when do Myocytes Die during Ischaemia and Reperfusion: The Late Phase'. *Journal of Cardiovascular Pharmacology and Therapeutics* 16 (3-4), 239-243

Baines, C. P. and Molkentin, J. D. (2005) 'STRESS Signaling Pathways that Modulate Cardiac Myocyte Apoptosis'. *Journal of Molecular and Cellular Cardiology* 38 (1), 47-62

Baines, C. P., Wang, L., Cohen, M. V., and Downey, J. M. (1999) 'Myocardial Protection by Insulin is Dependent on Phospatidylinositol 3-Kinase but Not Protein Kinase C Or KATP Channels in the Isolated Rabbit Heart'. *Basic Research in Cardiology* 94 (3), 188-198

Balint, G. A. (2001) 'Artemisinin and its Derivatives: An Important New Class of Antimalarial Agents'. *Pharmacology & Therapeutics* 90 (2–3), 261-265

Banek, K., Lalani, M., Staedke, S., and Chandramohan, D. (2014) 'Adherence to Artemisinin-Based Combination Therapy for the Treatment of Malaria: A Systematic Review of the Evidence'. *Malaria Journal* 13 (1), 7

Banerjee, N., Banerjee, M., Ganguly, S., Bandyopadhyay, S., Das, J. K., Bandyopadhay, A., Chatterjee, M., and Giri, A. K. (2008) 'Arsenic-Induced Mitochondrial Instability Leading to Programmed Cell Death in the Exposed Individuals'. *Toxicology* 246 (2–3), 101-111

Barrett, C. M., Lewis, F. L., Roaten, J. B., Sweatman, T. W., Israel, M., Cleveland, J. L., and Lothstein, L. (2002) 'Novel Extranuclear-Targeted Anthracyclines Override the Antiapoptotic Functions of Bcl-2 and Target Protein Kinase C Pathways to Induce Apoptosis'. *Molecular Cancer Therapeutics* 1 (7), 469-481

Barrett-Lee, P. J., Dixon, J. M., Farrell, C., Jones, A., Leonard, R., Murray, N., Palmieri, C., Plummer, C. J., Stanley, A., and Verrill, M. W. (2009) 'Expert Opinion on the use of Anthracyclines in Patients with Advanced Breast Cancer at Cardiac Risk'. *Annals of Oncology* 20 (5), 816-827

Barriere, C., Santamaria, D., Cerqueira, A., Galan, J., Martin, A., Ortega, S., Malumbres, M., Dubus, P., and Barbacid, M. (2007) 'Mice Thrive without Cdk4 and Cdk2'. *Molecular Oncology* 1 (1), 72-83

Barriere, J., Hoch, B., and Ferrero, J. M. (2012) 'New Perspectives in the Treatment of Metastatic Renal Cell Carcinoma'. *Critical Reviews in oncology/hematology* 84 Suppl 1, e16-23

Barros, L. F., Kanaseki, T., Sabirov, R., Morishima, S., Castro, J., Bittner, C. X., Maeno, E., Ando-Akatsuka, Y., and Okada, Y. (2003) 'Apoptotic and Necrotic Blebs in Epithelial Cells Display Similar Neck Diameters but Different Kinase Dependency'. *Cell Death and Differentiation* 10 (6), 687-697

Baxi, J., Barclay, C. J., and Gibbs, C. L. (2000) 'Energetics of Rat Papillary Muscle during Contractions with Sinusoidal Length Changes'. *American Journal of Physiology.Heart and Circulatory Physiology* 278 (5), H1545-54

Beekman, A. C., Woerdenbag, H. J., Kampinga, H. H., and Konings, A. W. T. (1996) 'Cytotoxicity of Artemisinin, a Dimer of Dihydroartemisinin, Artemisitene and

Eupatoriopicrin as Evaluated by the MTT and Clonogenic Assay'. *Phytotherapy Research* 10 (2), 140-144

Bell, R. M. and Yellon, D. M. (2003) 'Bradykinin Limits Infarction when Administered as an Adjunct to Reperfusion in Mouse Heart: The Role of PI3K, Akt and eNOS'. *Journal of Molecular and Cellular Cardiology* 35 (2), 185-193

BHF (2012).'Coronary heart disease statistics'. Available online from < https://www.bhf.org.uk/~/media/files/publications/research/2012_chd_statistics_compendium.pdf>

Bilia, A. R., Santomauro, F., Sacco, C., Bergonzi, M. C., and Donato, R. (2014) 'Essential Oil of Artemisia Annua L.: An Extraordinary Component with Numerous Antimicrobial Properties'. *Evidence-Based Complementary and Alternative Medicine: ECAM* 2014, 159819

Bird, B. R. J. H. and Swain, S. M. (2008) 'Cardiac Toxicity in Breast Cancer Survivors: Review of Potential Cardiac Problems'. *Clinical Cancer Research* 14 (1), 14-24

Blume-Jensen, P., Janknecht, R., and Hunter, T. (1998) 'The Kit Receptor Promotes Cell Survival Via Activation of PI 3-Kinase and Subsequent Akt-Mediated Phosphorylation of Bad on Ser136'. *Current Biology: CB* 8 (13), 779-782

Bolisetty, S. and Jaimes, E. A. (2013) 'Mitochondria and Reactive Oxygen Species: Physiology and Pathophysiology'. *International Journal of Molecular Sciences* 14 (3), 6306-6344

Bolli, R. (2001) 'Cardioprotective Function of Inducible Nitric Oxide Synthase and Role of Nitric Oxide in Myocardial Ischaemia and Preconditioning: An Overview of a Decade of Research'. *Journal of Molecular and Cellular Cardiology* 33 (11), 1897-1918

Bolli, R. (1990) 'Mechanism of Myocardial "Stunning".'. *Circulation* 82 (3), 723-738

Bonadonna, G. and Valagussa, P. (1981) 'Dose-Response Effect of Adjuvant Chemotherapy in Breast Cancer'. *N Engl J Med* 304 (1), 10-15

Bose, A. K., Mocanu, M. M., Carr, R. D., Brand, C. L., and Yellon, D. M. (2005) 'Glucagon-Like Peptide 1 can Directly Protect the Heart Against Ischemia/Reperfusion Injury'. *Diabetes* 54 (1), 146-151

Borutaite, V. and Brown, G. C. (2003) 'Mitochondria in Apoptosis of Ischemic Heart'. *FEBS Letters* 541 (1-3), 1-5

Borutaite, V., Jekabsone, A., Morkuniene, R., and Brown, G. C. (2003) 'Inhibition of Mitochondrial Permeability Transition Prevents Mitochondrial Dysfunction, Cytochrome c Release and Apoptosis Induced by Heart Ischaemia'. *Journal of Molecular and Cellular Cardiology* 35 (4), 357-366

Bose, A. K., Mocanu, M. M., Carr, R. D., and Yellon, D. M. (2007) 'Myocardial Ischaemia-Reperfusion Injury is Attenuated by Intact Glucagon Like Peptide-1 (GLP-1) in the in Vitro Rat Heart and may Involve the p70s6K Pathway'. *Cardiovascular Drugs and Therapy / Sponsored by the International Society of Cardiovascular Pharmacotherapy* 21 (4), 253-256

Bouchaud, O., Imbert, P., Touze, J. E., Dodoo, A. N., Danis, M., and Legros, F. (2009) 'Fatal Cardiotoxicity Related to Halofantrine: A Review Based on a Worldwide Safety Data Base'. *Malaria Journal* 8, 289-2875-8-289

Boultadakis, A. and Pitsikas, N. (2010) 'Effects of the Nitric Oxide Synthase Inhibitor L-NAME on Recognition and Spatial Memory Deficits Produced by Different NMDA Receptor Antagonists in the Rat'. *Neuropsychopharmacology: Official Publication of the American College of Neuropsychopharmacology* 35 (12), 2357-2366

Bourboulia, D. and Stetler-Stevenson, W. G. (2010) 'Matrix Metalloproteinases (MMPs) and Tissue Inhibitors of Metalloproteinases (TIMPs): Positive and Negative Regulators in Tumor Cell Adhesion'. *Seminars in Cancer Biology* 20 (3), 161-168

Bovelli, D., Plataniotis, G., Roila, F., and On behalf of the ESMO Guidelines Working Group (2010) 'Cardiotoxicity of Chemotherapeutic Agents and Radiotherapy-Related Heart Disease: ESMO Clinical Practice Guidelines'. *Annals of Oncology* 21 (suppl 5), v277-v282

Braunwald, E. and Kloner, R. A. (1985) 'Myocardial Reperfusion: A Double-Edged Sword?'. *The Journal of Clinical Investigation* 76 (5), 1713-1719

Braunwald, E. and Kloner, R. A. (1982) 'The Stunned Myocardium: Prolonged, Postischemic Ventricular Dysfunction'. *Circulation* 66 (6), 1146-1149

Brede, M., Roell, W., Ritter, O., Wiesmann, F., Jahns, R., Haase, A., Fleischmann, B. K., and Hein, L. (2003) 'Cardiac Hypertrophy is Associated with Decreased eNOS Expression in Angiotensin AT2 Receptor-Deficient Mice'. *Hypertension* 42 (6), 1177-1182

Bredt, D. S. and Snyder, S. H. (1994) 'Nitric Oxide: A Physiologic Messenger Molecule'. *Annual Review of Biochemistry* 63, 175-195

Brentnall, M., Rodriguez-Menocal, L., De Guevara, R. L., Cepero, E., and Boise, L. H. (2013) 'Caspase-9, Caspase-3 and Caspase-7 have Distinct Roles during Intrinsic Apoptosis'. *BMC Cell Biology* 14, 32-2121-14-32

Breivik, L., Jensen, A., Guvag, S., Aarnes, E. K., Aspevik, A., Helgeland, E., Hovland, S., Brattelid, T., and Jonassen, A. K. (2015) 'B-Type Natriuretic Peptide Expression and Cardioprotection is Regulated by Akt Dependent Signaling at Early Reperfusion'. Peptides 66, 43-50

Broder, H., Gottlieb, R. A., and Lepor, N. E. (2008) 'Chemotherapy and Cardiotoxicity'. *Reviews in Cardiovascular Medicine* 9 (2), 75-83

Brookes, P. S., Yoon, Y., Robotham, J. L., Anders, M. W., and Sheu, S. S. (2004) 'Calcium, ATP, and ROS: A Mitochondrial Love-Hate Triangle'. *American Journal of Physiology.Cell Physiology* 287 (4), C817-33

Brunelle, J. K. and Letai, A. (2009) 'Control of Mitochondrial Apoptosis by the Bcl-2 Family'. *Journal of Cell Science* 122 (Pt 4), 437-441

Bryan, N. S. and Grisham, M. B. (2007) 'Methods to Detect Nitric Oxide and its Metabolites in Biological Samples'. *Free Radical Biology & Medicine* 43 (5), 645-657

Buja, L. M. (2005) 'Myocardial Ischaemia and Reperfusion Injury'. *Cardiovascular Pathology* 14 (4), 170-175

Chua, C. C., Gao, J., Ho, Y. S., Xu, X., Kuo, I. C., Chua, K. Y., Wang, H., Hamdy, R. C., Reed, J. C., and Chua, B. H. (2009) 'Over-Expression of a Modified Bifunctional Apoptosis Regulator Protects Against Cardiac Injury and Doxorubicin-Induced Cardiotoxicity in Transgenic Mice'. *Cardiovascular Research* 81 (1), 20-27

Camara, A. K., Lesnefsky, E. J., and Stowe, D. F. (2010) 'Potential Therapeutic Benefits of Strategies Directed to Mitochondria'. *Antioxidants & Redox Signaling* 13 (3), 279-347

Capela, R., Oliveira, R., Goncalves, L. M., Domingos, A., Gut, J., Rosenthal, P. J., Lopes, F., and Moreira, R. (2009) 'Artemisinin-Dipeptidyl Vinyl Sulfone Hybrid Molecules: Design, Synthesis and Preliminary SAR for Antiplasmodial Activity and Falcipain-2 Inhibition'. *Bioorganic & Medicinal Chemistry Letters* 19 (12), 3229-3232

Carden, D. L. and Granger, D. N. (2000) 'Pathophysiology of Ischaemia-Reperfusion Injury'. *The Journal of Pathology* 190 (3), 255-266

Cardinale, D., Colombo, A., Lamantia, G., Colombo, N., Civelli, M., De Giacomi, G., Rubino, M., Veglia, F., Fiorentini, C., and Cipolla, C. M. (2010) 'Anthracycline-Induced Cardiomyopathy: Clinical Relevance and Response to Pharmacologic Therapy'. *Journal of the American College of Cardiology* 55 (3), 213-220

Care, A., Catalucci, D., Felicetti, F., Bonci, D., Addario, A., Gallo, P., Bang, M. L., Segnalini, P., Gu, Y., Dalton, N. D., Elia, L., Latronico, M. V., Hoydal, M., Autore, C., Russo, M. A., Dorn, G. W.,2nd, Ellingsen, O., Ruiz-Lozano, P., Peterson, K. L., Croce, C. M., Peschle, C., and Condorelli, G. (2007) 'MicroRNA-133 Controls Cardiac Hypertrophy'. *Nature Medicine* 13 (5), 613-618

Caserta, T. M., Smith, A. N., Gultice, A. D., Reedy, M. A., and Brown, T. L. (2003) 'Q-VD-OPh, a Broad Spectrum Caspase Inhibitor with Potent Antiapoptotic Properties'. *Apoptosis: An International Journal on Programmed Cell Death* 8 (4), 345-352

Cattaneo, M. G., Cappellini, E., Benfante, R., Ragni, M., Omodeo-Sale, F., Nisoli, E., Borgese, N., and Vicentini, L. M. (2011) 'Chronic Deficiency of Nitric Oxide Affects Hypoxia Inducible Factor-1alpha (HIF-1alpha) Stability and Migration in Human Endothelial Cells'. *PloS One* 6 (12), e29680

Caughey, G. E., Vitry, A. I., Gilbert, A. L., and Roughead, E. E. (2008) 'Prevalence of Comorbidity of Chronic Diseases in Australia'. *BMC Public Health* 8, 221-2458-8-221

Chadwick, J., Jones, M., Mercer, A. E., Stocks, P. A., Ward, S. A., Park, B. K., and O'Neill, P. M. (2010) 'Design, Synthesis and antimalarial/anticancer Evaluation of Spermidine Linked Artemisinin Conjugates Designed to Exploit Polyamine Transporters in Plasmodium Falciparum and HL-60 Cancer Cell Lines'. *Bioorganic & Medicinal Chemistry* 18 (7), 2586-2597

Challa, S. R., Akula, A., Metla, S., and Gopal, P. N. (2011) 'Partial Role of Nitric Oxide in Infarct Size Limiting Effect of Quercetin and Rutin Against Ischaemia-Reperfusion Injury in Normal and Diabetic Rats'. *Indian Journal of Experimental Biology* 49 (3), 207-210

Chaitanya, G. V., Steven, A. J., and Babu, P. P. (2010) 'PARP-1 Cleavage Fragments: Signatures of Cell-Death Proteases in Neurodegeneration'. *Cell Communication and Signaling: CCS* 8, 31-811X-8-31

Chakrabarti, S., Hoque, A. N., and Karmazyn, M. (1997) 'A Rapid Ischaemia-Induced Apoptosis in Isolated Rat Hearts and its Attenuation by the Sodium-Hydrogen Exchange Inhibitor HOE 642 (Cariporide)'. *Journal of Molecular and Cellular Cardiology* 29 (11), 3169-3174

Charras, G. T. (2008) 'A Short History of Blebbing'. *Journal of Microscopy* 231 (3), 466-478

Chatterjee, P.K., Patel, N.S.A., Kvale, E.O., Cuzzocrea, S., Brown, P.A.J., Stewart, K.N., Mota-Felipe, H. and Thiemermann, C. (2002) 'Inhibition of inducible nitric oxide synthase reduces renal ischemia/reperfusion injury' *Kidney International*, 61 (3), 862-871.

Chaturvedi, D., Goswami, A., Saikia, P. P., Barua, N. C., and Rao, P. G. (2010) 'Artemisinin and its Derivatives: A Novel Class of Anti-Malarial and Anti-Cancer Agents'. *Chemical Society Reviews* 39 (2), 435-454

Chauvier, D., Ankri, S., Charriaut-Marlangue, C., Casimir, R., and Jacotot, E. (2007) 'Broad-Spectrum Caspase Inhibitors: From Myth to Reality?' *Cell Death and Differentiation* 14 (2), 387-391

Chen, B., Peng, X., Pentassuglia, L., Lim, C. C., and Sawyer, D. B. (2007) 'Molecular and Cellular Mechanisms of Anthracycline Cardiotoxicity'. *Cardiovascular Toxicology* 7 (2), 114-121

Chen, H. H., Zhou, H. J., and Fang, X. (2003) 'Inhibition of Human Cancer Cell Line Growth and Human Umbilical Vein Endothelial Cell Angiogenesis by Artemisinin Derivatives in Vitro'. *Pharmacological Research: The Official Journal of the Italian Pharmacological Society* 48 (3), 231-236

Chen, J. F., Mandel, E. M., Thomson, J. M., Wu, Q., Callis, T. E., Hammond, S. M., Conlon, F. L., and Wang, D. Z. (2006) 'The Role of microRNA-1 and microRNA-133 in Skeletal Muscle Proliferation and Differentiation'. *Nature Genetics* 38 (2), 228-233

Chen, J., Long, J. B., Hurria, A., Owusu, C., Steingart, R. M., and Gross, C. P. (2012) 'Incidence of Heart Failure Or Cardiomyopathy After Adjuvant Trastuzumab Therapy for Breast Cancer'. *Journal of the American College of Cardiology* 60 (24), 2504-2512

Chen, P. S. and Tan, A. Y. (2007) 'Autonomic Nerve Activity and Atrial Fibrillation'. *Heart Rhythm: The Official Journal of the Heart Rhythm Society* 4 (3 Suppl), S61-4

Cheng, C., Ho, W. E., Goh, F. Y., Guan, S. P., Kong, L. R., Lai, W. Q., Leung, B. P., and Wong, W. S. (2011) 'Anti-Malarial Drug Artesunate Attenuates Experimental Allergic Asthma Via Inhibition of the Phosphoinositide 3-kinase/Akt Pathway'. *PloS One* 6 (6), e20932

Cheng, J. Q., Lindsley, C. W., Cheng, G. Z., Yang, H., and Nicosia, S. V. (2005) 'The Akt/PKB Pathway: Molecular Target for Cancer Drug Discovery'. *Oncogene* 24 (50), 7482

Cheng, H., Liu, W., and Ai, X. (2005) 'Protective Effect of Curcumin on Myocardial Ischemia Reperfusion Injury in Rats'. *Zhong Yao Cai = Zhongyaocai = Journal of Chinese Medicinal Materials* 28 (10), 920-922 7492

Cheng, J., Phong, B., Wilson, D. C., Hirsch, R., and Kane, L. P. (2011) *Akt Fine-Tunes NF-κB-Dependent Gene Expression during T Cell Activation*.

Cheng, R., Li, C., Li, C., Wei, L., Li, L., Zhang, Y., Yao, Y., Gu, X., Cai, W., Yang, Z., Ma, J., Yang, Xb., and Gao, G. (2013) 'The Artemisinin Derivative Artesunate Inhibits Corneal Neovascularization by Inducing ROS-Dependent Apoptosis in Vascular Endothelial Cells'. *Investigative Ophthalmology & Visual Science* 54 (5), 3400-3409

Cheng, Y., Tan, N., Yang, J., Liu, X., Cao, X., He, P., Dong, X., Qin, S., and Zhang, C. (2010) 'A Translational Study of Circulating Cell-Free microRNA-1 in Acute Myocardial Infarction'. *Clinical Science (London, England : 1979)* 119 (2), 87-95

Cheung, P., Sawicki, G., Wozniak, M., Wang, W., Radomski, M. W., and Schulz, R. (2000) 'Matrix Metalloproteinase-2 Contributes to Ischaemia-Reperfusion Injury in the Heart'. *Circulation* 101 (15), 1833-1839

Chhabra, R., Adlakha, Y. K., Hariharan, M., Scaria, V., and Saini, N. (2009) 'Upregulation of miR-23a-27a-24-2 Cluster Induces Caspase-Dependent and -Independent Apoptosis in Human Embryonic Kidney Cells'. *PloS One* 4 (6), e5848

Chi, X., Zhang, R., Shen, N., Jin, Y., Alina, A., Yang, S., and Lin, S. (2015) 'Sulforaphane Reduces Apoptosis and Oncosis Along with Protecting Liver Injury-Induced Ischemic Reperfusion by Activating the Nrf2/ARE Pathway'. *Hepatology International* 9 (2), 321-329

Chiang, C. W., Kanies, C., Kim, K. W., Fang, W. B., Parkhurst, C., Xie, M., Henry, T., and Yang, E. (2003) 'Protein Phosphatase 2A Dephosphorylation of Phosphoserine 112 Plays

the Gatekeeper Role for BAD-Mediated Apoptosis'. *Molecular and Cellular Biology* 23 (18), 6350-6362

Chiang, C. W., Harris, G., Ellig, C., Masters, S. C., Subramanian, R., Shenolikar, S., Wadzinski, B. E., and Yang, E. (2001) 'Protein Phosphatase 2A Activates the Proapoptotic Function of BAD in Interleukin- 3-Dependent Lymphoid Cells by a Mechanism Requiring 14-3-3 Dissociation'. *Blood* 97 (5), 1289-1297

Childs, A. C., Phaneuf, S. L., Dirks, A. J., Phillips, T., and Leeuwenburgh, C. (2002) 'Doxorubicin Treatment in Vivo Causes Cytochrome c Release and Cardiomyocyte Apoptosis, as Well as Increased Mitochondrial Efficiency, Superoxide Dismutase Activity, and Bcl-2:Bax Ratio'. *Cancer Research* 62 (16), 4592-4598

Chilton, R. J. (2004) 'Pathophysiology of Coronary Heart Disease: A Brief Review'. *JAOA: Journal of the American Osteopathic Association* 104 (9 suppl), 5S-8S

Chirino, Y. I., Trujillo, J., Sánchez-González, D. J., Martínez-Martínez, C. M., Cruz, C., Bobadilla, N. A., and Pedraza-Chaverri, J. (2008) 'Selective iNOS Inhibition Reduces Renal Damage Induced by Cisplatin'. *Toxicology Letters*176 (1), 48-57

Choi, B. M., Pae, H. O., Jang, S. I., Kim, Y. M., and Chung, H. T. (2002) 'Nitric Oxide as a Pro-Apoptotic as Well as Anti-Apoptotic Modulator'. *Journal of Biochemistry and Molecular Biology* 35 (1), 116-126

Chong, Z. Z., Li, F., and Maiese, K. (2005) 'Activating Akt and the Brain's Resources to Drive Cellular Survival and Prevent Inflammatory Injury'. *Histology and Histopathology* 20 (1), 299-315

Choy, E. H. and Panayi, G. S. (2001) 'Cytokine Pathways and Joint Inflammation in Rheumatoid Arthritis'. *The New England Journal of Medicine* 344 (12), 907-916

Chua, C. C., Gao, J., Ho, Y. S., Xu, X., Kuo, I. C., Chua, K. Y., Wang, H., Hamdy, R. C., Reed, J. C., and Chua, B. H. (2009) 'Over-Expression of a Modified Bifunctional Apoptosis

Regulator Protects Against Cardiac Injury and Doxorubicin-Induced Cardiotoxicity in Transgenic Mice'. *Cardiovascular Research* 81 (1), 20-27

Cole, M. P., Chaiswing, L., Oberley, T. D., Edelmann, S. E., Piascik, M. T., Lin, S. M., Kiningham, K. K., and St Clair, D. K. (2006) 'The Protective Roles of Nitric Oxide and Superoxide Dismutase in Adriamycin-Induced Cardiotoxicity'. *Cardiovascular Research* 69 (1), 186-197

Coleman, M. L., Sahai, E. A., Yeo, M., Bosch, M., Dewar, A., and Olson, M. F. (2001) 'Membrane Blebbing during Apoptosis Results from Caspase-Mediated Activation of ROCK I'. *Nature Cell Biology* 3 (4), 339-345

Collins, R. G., Velji, R., Guevara, N. V., Hicks, M. J., Chan, L., and Beaudet, A. L. (2000) 'P-Selectin Or Intercellular Adhesion Molecule (ICAM)-1 Deficiency Substantially Protects Against Atherosclerosis in Apolipoprotein E-Deficient Mice'. *The Journal of Experimental Medicine* 191 (1), 189-194

Collard, C. D. and Gelman, S. (2001) 'Pathophysiology, Clinical Manifestations, and Prevention of Ischaemia-Reperfusion Injury'. *Anesthesiology* 94 (6), 1133-1138

Conklin, K. A. (2005) 'Coenzyme q10 for Prevention of Anthracycline-Induced Cardiotoxicity'. *Integrative Cancer Therapies* 4 (2), 110-130

Corsten, M. F., Papageorgiou, A., Verhesen, W., Carai, P., Lindow, M., Obad, S., Summer, G., Coort, S. L., Hazebroek, M., van Leeuwen, R., Gijbels, M. J., Wijnands, E., Biessen, E. A., De Winther, M. P., Stassen, F. R., Carmeliet, P., Kauppinen, S., Schroen, B., and Heymans, S. (2012) 'MicroRNA Profiling Identifies microRNA-155 as an Adverse Mediator of Cardiac Injury and Dysfunction during Acute Viral Myocarditis'. *Circulation Research* 111 (4), 415-425

Courtney, K. D., Corcoran, R. B., and Engelman, J. A. (2010) 'The PI3K Pathway as Drug Target in Human Cancer'. *Journal of Clinical Oncology: Official Journal of the American Society of Clinical Oncology* 28 (6), 1075-1083

Crespo-Ortiz, M. P. and Wei, M. Q. (2012) 'Antitumor Activity of Artemisinin and its Derivatives: From a Well-Known Antimalarial Agent to a Potential Anticancer Drug'. *Journal of Biomedicine & Biotechnology* 2012, 247597

Crivellari, D., Bonetti, M., Castiglione-Gertsch, M., Gelber, R. D., Rudenstam, C. M., Thurlimann, B., Price, K. N., Coates, A. S., Hurny, C., Bernhard, J., Lindtner, J., Collins, J., Senn, H. J., Cavalli, F., Forbes, J., Gudgeon, A., Simoncini, E., Cortes-Funes, H., Veronesi, A., Fey, M., and Goldhirsch, A. (2000) 'Burdens and Benefits of Adjuvant Cyclophosphamide, Methotrexate, and Fluorouracil and Tamoxifen for Elderly Patients with Breast Cancer: The International Breast Cancer Study Group Trial VII'. *Journal of Clinical Oncology: Official Journal of the American Society of Clinical Oncology* 18 (7), 1412-1422

Crow, M. T., Mani, K., Nam, Y., and Kitsis, R. N. (2004) 'The Mitochondrial Death Pathway and Cardiac Myocyte Apoptosis'. *Circulation Research* 95 (10), 957-970

Csont T, Ferdinandy P (2005). Cardioprotective effects of glyceryl trinitrate: beyond vascular nitrate tolerance. *Pharmacol Ther* 105:57–68

Cui, H., Kong, Y., and Zhang, H. (2012) 'Oxidative Stress, Mitochondrial Dysfunction, and Aging'. *Journal of Signal Transduction* 2012, 646354

Cuzzocrea, S, Riley, D.P., Caputti, P. A and Salvemini, D (2001) ‘Antioxidant Therapy: A New Pharmacological Approach in Shock, Inflammation, and Ischemia/Reperfusion Injury’.Pharmacological Reviews 53 (1), 135-159

Da Costa Martins, P. A. and De Windt, L. J. (2012) 'MicroRNAs in Control of Cardiac Hypertrophy'. *Cardiovascular Research* 93 (4), 563-572

Dabbagh, A. and Rajaei, S. (2013) 'The Role of Anesthetic Drugs in Liver Apoptosis'. *Hepatitis Monthly* 13 (8), e13162

Dai, D. F., Chiao, Y. A., Marcinek, D. J., Szeto, H. H., and Rabinovitch, P. S. (2014) 'Mitochondrial Oxidative Stress in Aging and Healthspan'. *Longevity & Healthspan* 3, 6-2395-3-6. eCollection 2014

Das, A., Xi, L., and Kukreja, R. C. (2005) 'Phosphodiesterase-5 Inhibitor Sildenafil Preconditions Adult Cardiac Myocytes Against Necrosis and Apoptosis. Essential Role of Nitric Oxide Signaling'. *The Journal of Biological Chemistry* 280 (13), 12944-12955

D'Alessandra, Y., Pompilio, G., and Capogrossi, M. C. (2012) 'MicroRNAs and Myocardial Infarction'. *Current Opinion in Cardiology* 27 (3), 228-235

D'Alessandra, Y., Devanna, P., Limana, F., Straino, S., Di Carlo, A., Brambilla, P. G., Rubino, M., Carena, M. C., Spazzafumo, L., De Simone, M., Micheli, B., Biglioli, P., Achilli, F., Martelli, F., Maggiolini, S., Marenzi, G., Pompilio, G., and Capogrossi, M. C. (2010) 'Circulating microRNAs are New and Sensitive Biomarkers of Myocardial Infarction'. *European Heart Journal* 31 (22), 2765-2773

Danial, N. N. and Korsmeyer, S. J. (2004) 'Cell Death: Critical Control Points'. *Cell* 116 (2), 205-219

Daniel, P. T., Schulze-Osthoff, K., Belka, C., and Guner, D. (2003) 'Guardians of Cell Death: The Bcl-2 Family Proteins'. *Essays in Biochemistry* 39, 73-88

Daniels, T. R., Bernabeu, E., Rodriguez, J. A., Patel, S., Kozman, M., Chiappetta, D. A., Holler, E., Ljubimova, J. Y., Helguera, G., and Penichet, M. L. (2012) 'The Transferrin Receptor and the Targeted Delivery of Therapeutic Agents Against Cancer'. *Biochimica Et Biophysica Acta* 1820 (3), 291-317

Datta, S. R., Katsov, A., Hu, L., Petros, A., Fesik, S. W., Yaffe, M. B., and Greenberg, M. E. (2000) '14-3-3 Proteins and Survival Kinases Cooperate to Inactivate BAD by BH3 Domain Phosphorylation'. *Molecular Cell* 6 (1), 41-51

Davidson, S. M., Hausenloy, D., Duchen, M. R., and Yellon, D. M. (2006) 'Signalling Via the Reperfusion Injury Signalling Kinase (RISK) Pathway Links Closure of the

Mitochondrial Permeability Transition Pore to Cardioprotection'. *The International Journal of Biochemistry & Cell Biology* 38 (3), 414-419

Davis, T. M., Karunajeewa, H. A., and Ilett, K. F. (2005) 'Artemisinin-Based Combination Therapies for Uncomplicated Malaria'. *The Medical Journal of Australia* 182 (4), 181-185

de Bruin, E. C. and Medema, J. P. (2008) 'Apoptosis and Non-Apoptotic Deaths in Cancer Development and Treatment Response'. *Cancer Treatment Reviews* 34 (8), 737-749

del Peso, L., Gonzalez-Garcia, M., Page, C., Herrera, R., and Nunez, G. (1997) 'Interleukin-3-Induced Phosphorylation of BAD through the Protein Kinase Akt'. *Science (New York, N.Y.)* 278 (5338), 687-689

Diaz, R. (2000) 'Myocardial Protection during Acute Myocardial Infarction: The Need for a Simple Large Randomized Trial with GIK'. *Cardiovascular Drugs and Therapy / Sponsored by the International Society of Cardiovascular Pharmacotherapy* 14 (6), 561-563

Dickerson, F., Stallings, C., Vaughan, C., Origoni, A., Goga, J., Khushalani, S., and Yolken, R. (2011) 'Artemisinin Reduces the Level of Antibodies to Gliadin in Schizophrenia'. *Schizophrenia Research* 129 (2-3), 196-200

Dimmeler, S., Rippmann, V., Weiland, U., Haendeler, J., and Zeiher, A. M. (1997) 'Angiotensin II Induces Apoptosis of Human Endothelial Cells. Protective Effect of Nitric Oxide'. *Circulation Research* 81 (6), 970-976

Dimmeler, S. and Zeiher, A. M. (2010) 'Circulating microRNAs: Novel Biomarkers for Cardiovascular Diseases?'. *European Heart Journal* 31 (22), 2705-2707

Ding, W., Gu, J., Cao, L., Li, N., Ding, G., Wang, Z., Chen, L., Xu, X., and Xiao, W. 'Traditional Chinese Herbs as Chemical Resource Library for Drug Discovery of Anti-Infective and Anti-Inflammatory'. *Journal of Ethnopharmacology* (0)

Divakaran, V. and Mann, D. L. (2008) 'The Emerging Role of MicroRNAs in Cardiac Remodeling and Heart Failure'. *Circulation Research* 103 (10), 1072-1083

Doenst, T., Bothe, W., and Beyersdorf, F. (2003) 'Therapy with Insulin in Cardiac Surgery: Controversies and Possible Solutions'. *The Annals of Thoracic Surgery* 75 (2), S721-S728

Dondorp, A. M., Yeung, S., White, L., Nguon, C., Day, N. P., Socheat, D., and von Seidlein, L. (2010) 'Artemisinin Resistance: Current Status and Scenarios for Containment'. *Nature Reviews.Microbiology* 8 (4), 272-280

Dondorp, A. M., Fairhurst, R. M., Slutsker, L., MacArthur, J. R., M.D., J. G. B., Guerin, P. J., Wellems, T. E., Ringwald, P., Newman, R. D., and Plowe, C. V. (2011) 'The Threat of Artemisinin-Resistant Malaria'. *N Engl J Med* 365 (12), 1073-1075

Dondorp, A. M., Fairhurst, R. M., Slutsker, L., MacArthur, J. R., M.D., J. G. B., Guerin, P. J., Wellems, T. E., Ringwald, P., Newman, R. D., and Plowe, C. V. (2011) 'The Threat of Artemisinin-Resistant Malaria'. *N Engl J Med* 365 (12), 1073-1075

Downey, J.M. (1998) “Measuring infarct size by the tetrazolium method”. Available online from<http://www.usouthal.edu/ishr/help/ttc/>

Doyle, J. J., Neugut, A. I., Jacobson, J. S., Grann, V. R., and Hershman, D. L. (2005) 'Chemotherapy and Cardiotoxicity in Older Breast Cancer Patients: A Population-Based Study'. *Journal of Clinical Oncology: Official Journal of the American Society of Clinical Oncology* 23 (34), 8597-8605

Du, C., Fang, M., Li, Y., Li, L., and Wang, X. (2000) 'Smac, a Mitochondrial Protein that Promotes Cytochrome c–Dependent Caspase Activation by Eliminating IAP Inhibition'. *Cell* 102 (1), 33-42

Dudgeon, D. J., Kristjanson, L., Sloan, J. A., Lertzman, M., and Clement, K. (2001) 'Dyspnea in Cancer Patients: Prevalence and Associated Factors'. *Journal of Pain and Symptom Management* 21 (2), 95-102

du Toit, E. F., Genis, A., Opie, L. H., Pollesello, P., and Lochner, A. (2008) 'A Role for the RISK Pathway and K(ATP) Channels in Pre- and Post-Conditioning Induced by

Levosimendan in the Isolated Guinea Pig Heart'. *British Journal of Pharmacology* 154 (1), 41-50

Dunay, I. R., Chan, W. C., Haynes, R. K., and Sibley, L. D. (2009) 'Artemisone and Artemiside Control Acute and Reactivated Toxoplasmosis in a Murine Model'. *Antimicrobial Agents and Chemotherapy* 53 (10), 4450-4456

Efferth, T. and Kaina, B. (2010) 'Toxicity of the Antimalarial Artemisinin and its Dervatives'. *Critical Reviews in Toxicology* 40 (5), 405-421

Efferth, T. (2007) 'Willmar Schwabe Award 2006: Antiplasmodial and Antitumor Activity of Artemisinin--from Bench to Bedside'. *Planta Medica* 73 (4), 299-309

Efferth, T., Benakis, A., Romero, M. R., Tomicic, M., Rauh, R., Steinbach, D., Hafer, R., Stamminger, T., Oesch, F., Kaina, B., and Marschall, M. (2004) 'Enhancement of Cytotoxicity of Artemisinins Toward Cancer Cells by Ferrous Iron'. *Free Radical Biology & Medicine* 37 (7), 998-1009

Efferth, T., Briehl, M. M., and Tome, M. E. (2003) 'Role of Antioxidant Genes for the Activity of Artesunate Against Tumor Cells.'. *International Journal of Oncology* 23 (4), 1231-1235

Efferth, T., Romero, M. R., Wolf, D. G., Stamminger, T., Marin, J. J. G., and Marschall, M. (2008) 'The Antiviral Activities of Artemisinin and Artesunate'. *Clinical Infectious Diseases* 47 (6), 804-811

Efferth, T., Sauerbrey, A., Olbrich, A., Gebhart, E., Rauch, P., Weber, H. O., Hengstler, J. G., Halatsch, M., Volm, M., Tew, K. D., Ross, D. D., and Funk, J. O. (2003) *Molecular Modes of Action of Artesunate in Tumor Cell Lines*.

Elbaky NA, Ali AA, Ahmed RA. Cardioprotective effect of simvastatin on doxorubicininduced oxidative cardiotoxicity in rats. *Journal of Basic and Applied Sciences*. 2010;6(1):29–38

Elmore, S. (2007) 'Apoptosis: A Review of Programmed Cell Death'. *Toxicologic Pathology* 35 (4), 495-516

Eltzschig, H. K. and Eckle, T. (2011) 'Ischaemia and Reperfusion--from Mechanism to Translation'. *Nature Medicine* 17 (11), 1391-1401

Eltzschig, H. K. and Collard, C. D. (2004) 'Vascular Ischaemia and Reperfusion Injury'. *British Medical Bulletin* 70 (1), 71-86

Engelman, D. T., Watanabe, M., Engelman, R. M., Rousou, J. A., Kisin, E., Kagan, V. E., Maulik, N., and Das, D. K. (1995) 'Hypoxic Preconditioning Preserves Antioxidant Reserve in the Working Rat Heart'. *Cardiovascular Research* 29 (1), 133-140

Engelman JA, Luo J, Cantley LC. (2006) 'The evolution of phosphatidylinositol 3-kinases as regulators of growth and metabolism'. Nat Rev Genet 7(8):606-19.

Eschenhagen, T., Force, T., Ewer, M. S., de Keulenaer, G. W., Suter, T. M., Anker, S. D., Avkiran, M., de Azambuja, E., Balligand, J. L., Brutsaert, D. L., Condorelli, G., Hansen, A., Heymans, S., Hill, J. A., Hirsch, E., Hilfiker-Kleiner, D., Janssens, S., de Jong, S., Neubauer, G., Pieske, B., Ponikowski, P., Pirmohamed, M., Rauchhaus, M., Sawyer, D., Sugden, P. H., Wojta, J., Zannad, F., and Shah, A. M. (2011) 'Cardiovascular Side Effects of Cancer Therapies: A Position Statement from the Heart Failure Association of the European Society of Cardiology'. *European Journal of Heart Failure* 13 (1), 1-10

Ewer, M. S. and Ewer, S. M. (2010) 'Cardiotoxicity of Anticancer Treatments: What the Cardiologist Needs to Know'. *Nature Reviews.Cardiology* 7 (10), 564-575

Ewer, M. S., Vooletich, M. T., Durand, J. B., Woods, M. L., Davis, J. R., Valero, V., and Lenihan, D. J. (2005) 'Reversibility of Trastuzumab-Related Cardiotoxicity: New Insights Based on Clinical Course and Response to Medical Treatment'. *Journal of Clinical Oncology: Official Journal of the American Society of Clinical Oncology* 23 (31), 7820-7826

Fackler, O. T. and Grosse, R. (2008) 'Cell Motility through Plasma Membrane Blebbing'. *The Journal of Cell Biology* 181 (6), 879-884

Faghiri, Z. and Bazan, N. G. (2010) 'PI3K/Akt and mTOR/p70S6K Pathways Mediate Neuroprotectin D1-Induced Retinal Pigment Epithelial Cell Survival during Oxidative Stress-Induced Apoptosis'. *Experimental Eye Research*90 (6), 718-725

Fan, Y., Dickman, K. G., and Zong, W. X. (2010) 'Akt and c-Myc Differentially Activate Cellular Metabolic Programs and Prime Cells to Bioenergetic Inhibition'. *The Journal of Biological Chemistry* 285 (10), 7324-7333

Fan, Q., Chen, M., Zuo, L., Shang, X., Huang, M. Z., Ciccarelli, M., Raake, P., Brinks, H., Chuprun, K. J., Dorn, G. W.,2nd, Koch, W. J., and Gao, E. (2013) 'Myocardial Ablation of G Protein-Coupled Receptor Kinase 2 (GRK2) Decreases Ischemia/Reperfusion Injury through an Anti-Intrinsic Apoptotic Pathway'. *PloS One* 8 (6), e66234

Farah, C., Kleindienst, A., Bolea, G., Meyer, G., Gayrard, S., Geny, B., Obert, P., Cazorla, O., Tanguy, S., and Reboul, C. (2013) 'Exercise-Induced Cardioprotection: A Role for eNOS Uncoupling and NO Metabolites'. *Basic Research in Cardiology* 108 (6), 389-013-0389-2. Epub 2013 Oct 9

Felaco, M., Grilli, A., Gorbunov, N., Di Napoli, P., De Lutiis, M. A.,Di Guilio, C., Taccardi, A., Barsotti, A.,Barbacane, RC., Reale, M. and Conti, P. (2000) ‘Endothelial NOS expression and ischemia-reperfusion in isolated working rat heart from hypoxic and hyperoxic conditions’.*Biochem Biophys Acta* 15;1524(2-3):203-11.

Ferdinandy, P. (2006) 'Peroxynitrite: Just an oxidative/nitrosative Stressor Or a Physiological Regulator as Well?'. *British Journal of Pharmacology* 148 (1), 1-3

Ferdinandy, P., Schulz, R., and Baxter, G. F. (2007) 'Interaction of Cardiovascular Risk Factors with Myocardial Ischaemia/Reperfusion Injury, Preconditioning, and Postconditioning'. *Pharmacological Reviews* 59 (4), 418-458

Fernandes-Alnemri, T., Litwack, G., and Alnemri, E. S. (1994) 'CPP32, a Novel Human Apoptotic Protein with Homology to Caenorhabditis Elegans Cell Death Protein Ced-3 and Mammalian Interleukin-1 Beta-Converting Enzyme.' *Journal of Biological Chemistry* 269 (49), 30761-30764

Ferrari, R., Pepi, P., Ferrari, F., Nesta, F., Benigno, M., and Visioli, O. (1998) 'Metabolic Derangement in Ischemic Heart Disease and its Therapeutic Control'. *The American Journal of Cardiology* 82 (5A), 2K-13K

Ferri, K. F. and Kroemer, G. (2001) 'Organelle-Specific Initiation of Cell Death Pathways'. *Nature Cell Biology* 3 (11), E255-63

Feng, Q., Lu, X., Jones, D. L., Shen, J., and Arnold, J. M. (2001) 'Increased Inducible Nitric Oxide Synthase Expression Contributes to Myocardial Dysfunction and Higher Mortality After Myocardial Infarction in Mice'. *Circulation* 104 (6), 700-704

Feng, J., Bianchi, C., Sandmeyer, J. L., and Sellke, F. W. (2005) 'Bradykinin Preconditioning Improves the Profile of Cell Survival Proteins and Limits Apoptosis After Cardioplegic Arrest'. *Circulation* 112 (9 suppl), I-190-I-195

Fink, S. L. and Cookson, B. T. (2005) 'Apoptosis, Pyroptosis, and Necrosis: Mechanistic Description of Dead and Dying Eukaryotic Cells'. *Infection and Immunity* 73 (4), 1907-1916

Fisher, P. W., Salloum, F., Das, A., Hyder, H., and Kukreja, R. C. (2005) 'Phosphodiesterase-5 Inhibition with Sildenafil Attenuates Cardiomyocyte Apoptosis and Left Ventricular Dysfunction in a Chronic Model of Doxorubicin Cardiotoxicity'. *Circulation* 111 (13), 1601-1610

Fletcher, C. E., Dart, D. A., Sita-Lumsden, A., Cheng, H., Rennie, P. S., and Bevan, C. L. (2012) 'Androgen-Regulated Processing of the Oncomir miR-27a, which Targets Prohibitin in Prostate Cancer'. *Human Molecular Genetics* 21 (14), 3112-3127

Flobinus, A., Taudon, N., Desbordes, M., Labrosse, B., Simon, F., Mazeron, M. C., and Schnepf, N. (2014) 'Stability and Antiviral Activity Against Human Cytomegalovirus of Artemisinin Derivatives'. *The Journal of Antimicrobial Chemotherapy* 69 (1), 34-40

Foucrier, J., Bassaglia, Y., Grand, M. C., Rothen, B., Perriard, J. C., and Scherrer, K. (2001) 'Prosomes Form Sarcomere-Like Banding Patterns in Skeletal, Cardiac, and Smooth Muscle Cells'. *Experimental Cell Research* 266 (1), 193-200

Franciosa, J. A., Wilen, M., Ziesche, S., and Cohn, J. N. (1983) 'Survival in Men with Severe Chronic Left Ventricular Failure due to either Coronary Heart Disease Or Idiopathic Dilated Cardiomyopathy'. *The American Journal of Cardiology* 51 (5), 831-836

Franke, T. F., Hornik, C. P., Segev, L., Shostak, G. A., and Sugimoto, C. (2003) 'PI3K/Akt and Apoptosis: Size Matters'. *Oncogene* 22 (56), 8983-8998

Fulda, S., Gorman, A. M., Hori, O., and Samali, A. (2010) 'Cellular Stress Responses: Cell Survival and Cell Death'. *International Journal of Cell Biology* 2010, 214074

Fulda, S. and Debatin, K. M. (2006) 'Extrinsic Versus Intrinsic Apoptosis Pathways in Anticancer Chemotherapy'. *Oncogene* 25 (34), 4798-4811

Gabor A., B. (2001) 'Artemisinin and its Derivatives: An Important New Class of Antimalarial Agents'. *Pharmacology & Therapeutics* 90 (2–3), 261-265

Gao, T., Furnari, F., and Newton, A. C. (2005) 'PHLPP: A Phosphatase that Directly Dephosphorylates Akt, Promotes Apoptosis, and Suppresses Tumor Growth'. *Molecular Cell* 18 (1), 13-24

Gao, F., Gao, E., Yue, T., Ohlstein, E. H., Lopez, B. L., Christopher, T. A., and Ma, X. (2002) 'Nitric Oxide Mediates the Antiapoptotic Effect of Insulin in Myocardial Ischaemia-Reperfusion: The Roles of PI3-Kinase, Akt, and Endothelial Nitric Oxide Synthase Phosphorylation'. *Circulation* 105 (12), 1497-1502

Galagudza, M., Kurapeev, D., Minasian, S., Valen, G., and Vaage, J. (2004) 'Ischemic Postconditioning: Brief Ischemia during Reperfusion Converts Persistent Ventricular

Fibrillation into Regular Rhythm'. *European Journal of Cardio-Thoracic Surgery : Official Journal of the European Association for Cardio-Thoracic Surgery* 25 (6), 1006-1010

Geisberg, C. A. and Sawyer, D. B. (2010) 'Mechanisms of Anthracycline Cardiotoxicity and Strategies to Decrease Cardiac Damage'. *Current Hypertension Reports* 12 (6), 404-410

Gewirtz, D. A. (1999) 'A Critical Evaluation of the Mechanisms of Action Proposed for the Antitumor Effects of the Anthracycline Antibiotics Adriamycin and Daunorubicin'. *Biochemical Pharmacology* 57 (7), 727-741

Gharanei, M., Hussain, A., James, R. S., Janneh, O., and Maddock, H. (2014) 'Investigation into the Cardiotoxic Effects of Doxorubicin on Contractile Function and the Protection Afforded by Cyclosporin A using the Work-Loop Assay'. *Toxicology in Vitro: An International Journal Published in Association with BIBRA* 28 (5), 722-731

Gharanei, M., Hussain, A., Janneh, O., and Maddock, H. L. (2013) 'Doxorubicin Induced Myocardial Injury is Exacerbated Following Ischaemic Stress Via Opening of the Mitochondrial Permeability Transition Pore'. *Toxicology and Applied Pharmacology* 268 (2), 149-156

Ghosh, S., Ng, L. L., Talwar, S., Squire, I. B., and Galinanes, M. (2000) 'Cardiotrophin-1 Protects the Human Myocardium from Ischemic Injury. Comparison with the First and Second Window of Protection by Ischemic Preconditioning'. *Cardiovascular Research* 48 (3), 440-447

Gianni, L., Herman, E. H., Lipshultz, S. E., Minotti, G., Sarvazyan, N., and Sawyer, D. B. (2008) 'Anthracycline Cardiotoxicity: From Bench to Bedside'. *Journal of Clinical Oncology: Official Journal of the American Society of Clinical Oncology* 26 (22), 3777-3784

Gibson, L. M., Brazzelli, M., Thomas, B. M., and Sandercock, P. A. (2010) 'A Systematic Review of Clinical Trials of Pharmacological Interventions for Acute Ischaemic Stroke (1955-2008) that were Completed, but Not Published in Full'. *Trials* 11, 43-6215-11-43

Gidlof, O., Andersson, P., van der Pals, J., Gotberg, M., and Erlinge, D. (2011) 'Cardiospecific microRNA Plasma Levels Correlate with Troponin and Cardiac Function in Patients with ST Elevation Myocardial Infarction, are Selectively Dependent on Renal Elimination, and can be Detected in Urine Samples'. *Cardiology* 118 (4), 217-226

Gilleron, M., Marechal, X., Montaigne, D., Franczak, J., Neviere, R., and Lancel, S. (2009) 'NADPH Oxidases Participate to Doxorubicin-Induced Cardiac Myocyte Apoptosis'. *Biochemical and Biophysical Research Communications*388 (4), 727-731

Glick, D., Barth, S., and Macleod, K. F. (2010) 'Autophagy: Cellular and Molecular Mechanisms'. *The Journal of Pathology* 221 (1), 3-12

Golenser, J., Waknine, J. H., Krugliak, M., Hunt, N. H., and Grau, G. E. (2006) 'Current Perspectives on the Mechanism of Action of Artemisinins'. *International Journal for Parasitology* 36 (14), 1427-1441

Gordon, J. M., Dusting, G. J., Woodman, O. L., and Ritchie, R. H. (2003) 'Cardioprotective Action of CRF Peptide Urocortin Against Simulated Ischaemia in Adult Rat Cardiomyocytes'. *American Journal of Physiology.Heart and Circulatory Physiology* 284 (1), H330-6

Gottlieb, R. A., Burleson, K. O., Kloner, R. A., Babior, B. M., and Engler, R. L. (1994) 'Reperfusion Injury Induces Apoptosis in Rabbit Cardiomyocytes'. *The Journal of Clinical Investigation* 94 (4), 1621-1628

Gown, A. M. and Willingham, M. C. (2002) 'Improved Detection of Apoptotic Cells in Archival Paraffin Sections: Immunohistochemistry using Antibodies to Cleaved Caspase 3'. *Journal of Histochemistry & Cytochemistry* 50 (4), 449-454

Graham M. M, Faris P. D, Ghali W. A, et al. (2001) ‘Validation of three myocardial jeopardy scores in a population-based cardiac catheterization cohort’. Am Heart J. 142:254–61

Gross, G. J. (2003) 'Role of Opioids in Acute and Delayed Preconditioning'. *Journal of Molecular and Cellular Cardiology* 35 (7), 709-718

Grundy, S. M. (1999) 'Primary Prevention of Coronary Heart Disease: Integrating Risk Assessment with Intervention'. *Circulation* 100 (9), 988-998

Goldberg, R. J., Spencer, F. A., Yarzebski, J., Lessard, D., Gore, J. M., Alpert, J. S., and Dalen, J. E. (2004) 'A 25-Year Perspective into the Changing Landscape of Patients Hospitalized with Acute Myocardial Infarction (the Worcester Heart Attack Study)'. *The American Journal of Cardiology* 94 (11), 1373-1378

Gu, Y., Wang, X., Yuan, M., Wu, G., Hu, J., Tang, Y and Huang, C (2012) 'Artemisinin Attenuates Post-Infarct Myocardial Remodelling by Down-Regulating the NF-κB Pathway'.*Tohoku J Exp Med* 227,161-170.

Gutierrez, F. R., Mineo, T. W., Pavanelli, W. R., Guedes, P. M., and Silva, J. S. (2009) 'The Effects of Nitric Oxide on the Immune System during Trypanosoma Cruzi Infection'. *Memorias do Instituto Oswaldo Cruz* 104 Suppl 1, 236-245

Gupta, R. K., Van Vugt, M., Paiphun, L., Slight, T., Looarersuwan, S., Whit, N. J., and Nosten, F. (2005) 'No Evidence of Cardiotoxicity of Atovaquone-Proguanil Alone Or in Combination with Artesunate'. *The American Journal of Tropical Medicine and Hygiene* 73 (2), 267-268

Ha, H. C. and Snyder, S. H. (2000) 'Poly (ADP-Ribose) Polymerase-1 in the Nervous System'. *Neurobiology of Disease* 7 (4), 225-239

Ha, T. Y. (2011) 'MicroRNAs in Human Diseases: From Cancer to Cardiovascular Disease'. *Immune Network* 11 (3), 135-154

Ha, M. and Kim, V. N. (2014) 'Regulation of microRNA Biogenesis'. *Nature Reviews.Molecular Cell Biology* 15 (8), 509-524

Hadjipanayi, E. and Schilling, A. F. (2013) 'Hypoxia-Based Strategies for Angiogenic Induction: The Dawn of a New Era for Ischaemia Therapy and Tissue Regeneration'. *Organogenesis* 9 (4), 261-272

Halestrap, A. P. and Richardson, A. P. (2015) 'The Mitochondrial Permeability Transition: A Current Perspective on its Identity and Role in ischaemia/reperfusion Injury'. *Journal of Molecular and Cellular Cardiology* 78, 129-141

Halestrap, A. P., Clarke, S. J., and Javadov, S. A. (2004) 'Mitochondrial Permeability Transition Pore Opening during Myocardial Reperfusion--a Target for Cardioprotection'. *Cardiovascular Research* 61 (3), 372-385

Halestrap, A. P. and Brenner, C. (2003) 'The Adenine Nucleotide Translocase: A Central Component of the Mitochondrial Permeability Transition Pore and Key Player in Cell Death'. *Current Medicinal Chemistry* 10 (16), 1507-1525

Halestrap, A. P. and Pasdois, P. (2009) 'The Role of the Mitochondrial Permeability Transition Pore in Heart Disease'. *Biochimica Et Biophysica Acta (BBA) - Bioenergetics* 1787 (11), 1402-1415

Halestrap, A. P. and Richardson, A. P. (2015) 'The Mitochondrial Permeability Transition: A Current Perspective on its Identity and Role in ischaemia/reperfusion Injury'. *Journal of Molecular and Cellular Cardiology* 78 (0), 129-141

Halladin, N. L. (2015) 'Oxidative and Inflammatory Biomarkers of Ischaemia and Reperfusion Injuries'. *Danish Medical Journal* 62 (4), B5054

Hamacher-Brady, A., Stein, H. A., Turschner, S., Toegel, I., Mora, R., Jennewein, N., Efferth, T., Eils, R., and Brady, N. R. (2011) 'Artesunate Activates Mitochondrial Apoptosis in Breast Cancer Cells Via Iron-Catalyzed Lysosomal Reactive Oxygen Species Production'. *The Journal of Biological Chemistry* 286 (8), 6587-6601

Han, J., Pearson, R. B., Dennis, P. B., and Thomas, G. (1995) 'Rapamycin, Wortmannin, and the Methylxanthine SQ20006 Inactivate p70s6k by Inducing Dephosphorylation of the Same Subset of Sites'. *Journal of Biological Chemistry* 270 (36), 21396-21403

Han, S. Y., Li, H. X., Ma, X., Zhang, K., Ma, Z. Z., and Tu, P. F. (2009) 'Protective Effects of Purified Safflower Extract on Myocardial Ischemia in Vivo and in Vitro'. *Phytomedicine : International Journal of Phytotherapy and Phytopharmacology* 16 (8), 694-702

Hancock, J. T., Desikan, R., and Neill, S. J. (2001) 'Role of Reactive Oxygen Species in Cell Signalling Pathways'. *Biochemical Society Transactions* 29 (Pt 2), 345-350

Handa, H. (2014) 'New Treatment and Diagnostic Test for Multiple Myeloma'. *Rinsho Byori.the Japanese Journal of Clinical Pathology* 62 (4), 337-351

Hara, Y., Yamawaki, H., Shimada, M., Okada, K., Tanai, T., Ichikawa, D., Miyake, K., and Kizaki, K. (2007) 'Anticholinergic Effects of Artemisinin, an Antimalarial Drug, in Isolated Guinea Pig Heart Preparations'. *The Journal of Veterinary Medical Science / the Japanese Society of Veterinary Science* 69 (7), 697-702

Hara, Y. and Kizaki, K. (2002) 'Antimalarial Drugs Inhibit the Acetylcholine-Receptor-Operated Potassium Current in Atrial Myocytes'. *Heart, Lung & Circulation* 11 (2), 112-116

Harada, H., Andersen, J. S., Mann, M., Terada, N., and Korsmeyer, S. J. (2001) 'P70S6 Kinase Signals Cell Survival as Well as Growth, Inactivating the Pro-Apoptotic Molecule BAD'. *Proceedings of the National Academy of Sciences of the United States of America* 98 (17), 9666-9670

Harake, D., Franco, V. I., Henkel, J. M., Miller, T. L., and Lipshultz, S. E. (2012) 'Cardiotoxicity in Childhood Cancer Survivors: Strategies for Prevention and Management'. *Future Cardiology* 8 (4), 647-670

Harter, G. and Michel, D. (2012) 'Antiviral Treatment of Cytomegalovirus Infection: An Update'. *Expert Opinion on Pharmacotherapy* 13 (5), 623-627

Hausenloy, D. J. and Yellon, D. M. (2013) 'Myocardial Ischaemia-Reperfusion Injury: A Neglected Therapeutic Target'. *The Journal of Clinical Investigation* 123 (1), 92-100

Hausenloy, D. J., Tsang, A., Mocanu, M. M., and Yellon, D. M. (2005) 'Ischemic Preconditioning Protects by Activating Prosurvival Kinases at Reperfusion'. *American Journal of Physiology.Heart and Circulatory Physiology* 288 (2), H971-6

Hausenloy, D. J. and Yellon, D. M. (2004) 'New Directions for Protecting the Heart Against ischaemia–reperfusion Injury: Targeting the Reperfusion Injury Salvage Kinase (RISK)-Pathway'. *Cardiovascular Research* 61 (3), 448-460

He, B., Xiao, J., Ren, A. J., Zhang, Y. F., Zhang, H., Chen, M., Xie, B., Gao, X. G., and Wang, Y. W. (2011) 'Role of miR-1 and miR-133a in Myocardial Ischemic Postconditioning'. *Journal of Biomedical Science* 18, 22-0127-18-22

He, L. and Hannon, G. J. (2004) 'MicroRNAs: Small RNAs with a Big Role in Gene Regulation'. *Nature Reviews.Genetics* 5 (7), 522-531

He, L. and Lemasters, J. J. (2002) 'Regulated and Unregulated Mitochondrial Permeability Transition Pores: A New Paradigm of Pore Structure and Function?'. *FEBS Letters* 512 (1–3), 1-7

Healy, D., Clarke-Moloney, M., Gaughan, B., O'Daly, S., Hausenloy, D., Sharif, F., Newell, J., O'Donnell, M., Grace, P., Forbes, J. F., Cullen, W., Kavanagh, E., Burke, P., Cross, S., Dowdall, J., McMonagle, M., Fulton, G., Manning, B. J., Kheirelseid, E. A., Leahy, A., Moneley, D., Naughton, P., Boyle, E., McHugh, S., Madhaven, P., O'Neill, S., Martin, Z., Courtney, D., Tubassam, M., Sultan, S., McCartan, D., Medani, M., and Walsh, S. (2015) 'Preconditioning Shields Against Vascular Events in Surgery (SAVES), a Multicentre Feasibility Trial of Preconditioning Against Adverse Events in Major Vascular Surgery: Study Protocol for a Randomised Control Trial'. *Trials* 16 (1), 185-015-0678-1

Hemmings, B. A. and Restuccia, D. F. (2012) 'PI3K-PKB/Akt Pathway'. *Cold Spring Harbor Perspectives in Biology* 4 (9), a011189

Hengartner, M. O. (2000) 'The Biochemistry of Apoptosis'. *Nature* 407 (6805), 770-776

Heusch, G. (2008) 'HR in the Pathophysiology of Coronary Blood Flow and Myocardial Ischaemia: Benefit from Selective Bradycardic Agents'. *British Journal of Pharmacology* 153 (8), 1589-1601

Heusch, G., Boengler, K., and Schulz, R. (2008) 'Cardioprotection: Nitric Oxide, Protein Kinases, and Mitochondria'. *Circulation* 118 (19), 1915-1919

Hiona, A., Lee, A. S., Nagendran, J., Xie, X., Connolly, A. J., Robbins, R. C., and Wu, J. C. (2011) 'Pretreatment with Angiotensin-Converting Enzyme Inhibitor Improves Doxorubicin-Induced Cardiomyopathy Via Preservation of Mitochondrial Function'. *The Journal of Thoracic and Cardiovascular Surgery* 142 (2), 396-403.e3

Ho, W. E., Peh, H. Y., Chan, T. K., and Wong, W. S. (2014) 'Artemisinins: Pharmacological Actions Beyond Anti-Malarial'. *Pharmacology & Therapeutics* 142 (1), 126-139

Ho, W. E., Cheng, C., Peh, H. Y., Xu, F., Tannenbaum, S. R., Ong, C. N., and Wong, W. S. F. (2012) 'Anti-Malarial Drug Artesunate Ameliorates Oxidative Lung Damage in Experimental Allergic Asthma'. *Free Radical Biology and Medicine* 53 (3), 498-507

Hoshikawa, Y., Ono, S., Suzuki, S., Tanita, T., Chida, M., Song, C., Noda, M., Tabata, T., Voelkel, N. F., and Fujimura, S. (2001) 'Generation of Oxidative Stress Contributes to the Development of Pulmonary Hypertension Induced by Hypoxia'. *Journal of Applied Physiology (Bethesda, Md.: 1985)* 90 (4), 1299-1306

Hou, J., Wang, D., Zhang, R., and Wang, H. (2008) 'Experimental Therapy of Hepatoma with Artemisinin and its Derivatives: In Vitro and in Vivo Activity, Chemosensitization, and Mechanisms of Action'. *Clinical Cancer Research: An Official Journal of the American Association for Cancer Research* 14 (17), 5519-5530

Hou, L., Block, K. E., and Huang, H. (2014) 'Artesunate Abolishes Germinal Center B Cells and Inhibits Autoimmune Arthritis'. *PloS One* 9 (8), e104762

Hussain, A., Gharanei, A. M., Nagra, A. S., and Maddock, H. L. (2014) 'Caspase Inhibition Via A3 Adenosine Receptors: A New Cardioprotective Mechanism Against Myocardial

Infarction'. *Cardiovascular Drugs and Therapy / Sponsored by the International Society of Cardiovascular Pharmacotherapy* 28 (1), 19-32

Hussain, A., Maddock, H., Al-Rajaibi, H., and Carson, R. J. (2011) 'Effects of Hydrogen Sulphide on the Isolated Perfused Rat Heart'. *Sultan Qaboos University Medical Journal* 11 (2), 236-244

Ibáñez B, Heusch G, Ovize M, Van de Werf F. Evolving Therapies for Myocardial Ischemia/Reperfusion Injury. J Am Coll Cardiol. 2015; 65(14):1454-1471. doi:10.1016/j.jacc.2015.02.032

Ibrahim, N. A., Bjornsdottir, I., Al Alwan, A. S., and Honore, P. H. (2013) 'Insights about Health-Related Quality of Life in Cancer Patients Indicate Demands for Better Pharmaceutical Care'. *Journal of Oncology Pharmacy Practice : Official Publication of the International Society of Oncology Pharmacy Practitioners* 20 (4), 270-277

Ichikawa, Y., Ghanefar, M., Bayeva, M., Wu, R., Khechaduri, A., Naga Prasad, S. V., Mutharasan, R. K., Naik, T. J., and Ardehali, H. (2014) 'Cardiotoxicity of Doxorubicin is Mediated through Mitochondrial Iron Accumulation'. *The Journal of Clinical Investigation* 124 (2), 617-630

Ikeda, S., Kong, S. W., Lu, J., Bisping, E., Zhang, H., Allen, P. D., Golub, T. R., Pieske, B., and Pu, W. T. (2007) 'Altered microRNA Expression in Human Heart Disease'. *Physiological Genomics* 31 (3), 367-373

Iliodromitis, E. K., Lazou, A., and Kremastinos, D. T. (2007) 'Ischemic Preconditioning: Protection Against Myocardial Necrosis and Apoptosis'. *Vascular Health and Risk Management* 3 (5), 629-637

Imazio, M. and Adler, Y. (2013) 'Treatment with Aspirin, NSAID, Corticosteroids, and Colchicine in Acute and Recurrent Pericarditis'. *Heart Failure Reviews* 18 (3), 355-360

Indran, I. R., Tufo, G., Pervaiz, S., and Brenner, C. (2011) 'Recent Advances in Apoptosis, Mitochondria and Drug Resistance in Cancer Cells'. *Biochimica Et Biophysica Acta (BBA) - Bioenergetics* 1807 (6), 735-745

Inomata, N., Ohno, T., Ishihara, T., and Akaike, N. (1993) 'Antiarrhythmic Agents Act Differently on the Activation Phase of the ACh-Response in Guinea-Pig Atrial Myocytes'. *British Journal of Pharmacology* 108 (1), 111-115

Isenovic, E., Soskic, S., Dungen, H. D., Dobutovic, B., Elvis, T., Simone, I., and Marche, P. (2011) 'Regulation of Endothelial Nitric Oxide Synthase in Pathophysiological Conditions'. *Cardiovascular & Hematological Disorders Drug Targets*

Jankov, R. P., Kantores, C., Pan, J., and Belik, J. (2008) 'Contribution of Xanthine Oxidase-Derived Superoxide to Chronic Hypoxic Pulmonary Hypertension in Neonatal Rats'. *American Journal of Physiology.Lung Cellular and Molecular Physiology* 294 (2), L233-45

Janssens, V. and Goris, J. (2001) 'Protein Phosphatase 2A: A Highly Regulated Family of serine/threonine Phosphatases Implicated in Cell Growth and Signalling'. *The Biochemical Journal* 353 (Pt 3), 417-439

Javadov, S., Karmazyn, M., and Escobales, N. (2009) 'Mitochondrial Permeability Transition Pore Opening as a Promising Therapeutic Target in Cardiac Diseases'. *Journal of Pharmacology and Experimental Therapeutics* 330 (3), 670-678

Jiang, Y., Wang, H. Y., Li, Y., Guo, S. H., Zhang, L., and Cai, J. H. (2014) 'Peripheral Blood miRNAs as a Biomarker for Chronic Cardiovascular Diseases'. *Scientific Reports* 4, 5026

Jiang, X. and Wang, X. (2000) 'Cytochrome c Promotes Caspase-9 Activation by Inducing Nucleotide Binding to Apaf-1'. *Journal of Biological Chemistry* 275 (40), 31199-31203

Juneja, D., Singh, O., Bhasin, A., Gupta, M., Saxena, S., and Chaturvedi, A. (2012) 'Severe Suicidal Digoxin Toxicity Managed with Resin Hemoperfusion: A Case Report'. *Indian*

Journal of Critical Care Medicine : Peer-Reviewed, Official Publication of Indian Society of Critical Care Medicine 16 (4), 231-233

Jugdutt, B. I. (2003) 'Nitric Oxide and Cardiovascular Protection'. *Heart Failure Reviews* 8 (1), 29-34

Johansson, C., Magnusson, O., Deveney, A. M., Jackson, D. M., Zhang, J., Engel, J. A., and Svensson, L. (1998) 'The Nitric Oxide Synthase Inhibitor. L-NAME, Blocks Certain Phencyclidine-Induced but Not Amphetamine-Induced Effects on Behaviour and Brain Biochemistry in the Rat'. Progress in Neuro-Psychopharmacology and Biological Psychiatry 22 (8), 1341-1360

Johnson, R. A., Wang, X., Ma, X. L., Huong, S. M., and Huang, E. S. (2001) 'Human Cytomegalovirus Up-Regulates the Phosphatidylinositol 3-Kinase (PI3-K) Pathway: Inhibition of PI3-K Activity Inhibits Viral Replication and Virus-Induced Signaling'. *Journal of Virology* 75 (13), 6022-6032

Jonassen, A. K., Mjøs, O. D., and Sack, M. N. (2004) 'P70s6 Kinase is a Functional Target of Insulin Activated Akt Cell-Survival Signaling'. *Biochemical and Biophysical Research Communications* 315 (1), 160-165

Jones, M., Mercer, A. E., Stocks, P. A., La Pensee, L. J., Cosstick, R., Park, B. K., Kennedy, M. E., Piantanida, I., Ward, S. A., Davies, J., Bray, P. G., Rawe, S. L., Baird, J., Charidza, T., Janneh, O., and O'Neill, P. M. (2009) 'Antitumour and Antimalarial Activity of Artemisinin-Acridine Hybrids'. *Bioorganic & Medicinal Chemistry Letters* 19 (7), 2033-2037

Jones, S. P. and Bolli, R. (2006) 'The Ubiquitous Role of Nitric Oxide in Cardioprotection'. *Journal of Molecular and Cellular Cardiology* 40 (1), 16-23

Jovanovic, D. V., Mineau, F., Notoya, K., Reboul, P., Martel-Pelletier, J., and Pelletier, J. P. (2002) 'Nitric Oxide Induced Cell Death in Human Osteoarthritic Synoviocytes is Mediated by Tyrosine Kinase Activation and Hydrogen Peroxide and/or Superoxide Formation'. *The Journal of Rheumatology* 29 (10), 2165-2175

Kane, L. P. and Weiss, A. (2003) 'The PI-3 kinase/Akt Pathway and T Cell Activation: Pleiotropic Pathways Downstream of PIP3'. *Immunological Reviews* 192, 7-20

Kajihara, H., Yokozaki, H., Yamahara, M., Kadomoto, Y., and Tahara, E. (1986) 'Anthracycline Induced Myocardial Damage. an Analysis of 16 Autopsy Cases'. *Pathology, Research and Practice* 181 (4), 434-441

Kajstura, J., Cheng, W., Reiss, K., Clark, W. A., Sonnenblick, E. H., Krajewski, S., Reed, J. C., Olivetti, G., and Anversa, P. (1996) 'Apoptotic and Necrotic Myocyte Cell Deaths are Independent Contributing Variables of Infarct Size in Rats'. *Laboratory Investigation; a Journal of Technical Methods and Pathology* 74 (1), 86-107

Kalogeris, T., Baines, C. P., Krenz, M., and Korthuis, R. J. (2012) 'Cell Biology of ischaemia/reperfusion Injury'. *International Review of Cell and Molecular Biology* 298, 229-317

Kalivendi, S. V., Kotamraju, S., Zhao, H., Joseph, J., and Kalyanaraman, B. (2001) 'Doxorubicin-Induced Apoptosis is Associated with Increased Transcription of Endothelial Nitric-Oxide Synthase. Effect of Antiapoptotic Antioxidants and Calcium'. *The Journal of Biological Chemistry* 276 (50), 47266-47276

Kang, Y. J., Chen, Y., Yu, A., Voss-McCowan, M., and Epstein, P. N. (1997) 'Overexpression of Metallothionein in the Heart of Transgenic Mice Suppresses Doxorubicin Cardiotoxicity'. *The Journal of Clinical Investigation* 100 (6), 1501-1506

Kang, K. A., Wang, Z. H., Zhang, R., Piao, M. J., Kim, K. C., Kang, S. S., Kim, Y. W., Lee, J., Park, D., and Hyun, J. W. (2010) 'Myricetin Protects Cells Against Oxidative Stress-Induced Apoptosis Via Regulation of PI3K/Akt and MAPK Signaling Pathways'. *International Journal of Molecular Sciences* 11 (11), 4348-4360

Kanno, S., Lee, P. C., Zhang, Y., Ho, C., Griffith, B. P., Shears, L. L., and Billiar, T. R. (2000) 'Attenuation of Myocardial Ischaemia/Reperfusion Injury by Superinduction of Inducible Nitric Oxide Synthase'. *Circulation* 101 (23), 2742-2748

Kapakos, G., Bouallegue, A., Daou, G. B., and Srivastava, A. K. (2010) 'Modulatory Role of Nitric Oxide/cGMP System in Endothelin-1-Induced Signaling Responses in Vascular Smooth Muscle Cells'. *Current Cardiology Reviews* 6 (4), 247-254

Kar, K., Nath, A., Bajpai, R., Dutta, G. P., and Vishwakarma, R. A. (1989) 'Pharmacology of alpha/beta Arteether--a Potential Antimalarial Drug'. *Journal of Ethnopharmacology* 27 (3), 297-305

Kaushal, G. P., Kaushal, V., Hong, X., and Shah, S. V. (2001) 'Role and Regulation of Activation of Caspases in Cisplatin-Induced Injury to Renal Tubular Epithelial Cells'. *Kidney International* 60 (5), 1726-1736

Keefe, D. L. (2001) 'Anthracycline-Induced Cardiomyopathy'. *Seminars in Oncology* 28 (4 Suppl 12), 2-7

Kell, D. B. (2009) 'Iron Behaving Badly: Inappropriate Iron Chelation as a Major Contributor to the Aetiology of Vascular and Other Progressive Inflammatory and Degenerative Diseases'. *BMC Medical Genomics* 2, 2-8794-2-2

Kemp, M., Donovan, J., Higham, H., and Hooper, J. (2004) 'Biochemical Markers of Myocardial Injury'. *British Journal of Anaesthesia* 93 (1), 63-73

Keoni, C. L. and Brown, T. L. (2015) 'Inhibition of Apoptosis and Efficacy of Pan Caspase Inhibitor, Q-VD-OPh, in Models of Human Disease'. *Journal of Cell Death* 8, 1-7

Kidd, L. B., Schabbauer, G. A., Luyendyk, J. P., Holscher, T. D., Tilley, R. E., Tencati, M., and Mackma, N. (2008) 'Insulin Activation of the Phosphatidylinositol 3-Kinase/Protein Kinase B (Akt) Pathway Reduces Lipopolysaccharide-Induced Inflammation in Mice'. *Journal of Pharmacology and Experimental Therapeutics* 326 (1), 348-353

Kim, N. H. and Kang, P. M. (2010) 'Apoptosis in Cardiovascular Diseases: Mechanism and Clinical Implications'. *Korean Circulation Journal* 40 (7), 299-305

Kim, S. H., Kim, H. J., and Kim, T. S. (2003) 'Differential Involvement of Protein Kinase C in Human Promyelocytic Leukemia Cell Differentiation Enhanced by Artemisinin'. *European Journal of Pharmacology* 482 (1-3), 67-76

Kim, P. K. M., Zamora, R., Petrosko, P., and Billiar, T. R. (2001) 'The Regulatory Role of Nitric Oxide in Apoptosis'. *International Immunopharmacology* 1 (8), 1421-1441

Kim, S. Y., Kim, S. J., Kim, B. J., Rah, S. Y., Chung, S. M., Im, M. J., and Kim, U. H. (2006) 'Doxorubicin-Induced Reactive Oxygen Species Generation and Intracellular Ca2+ Increase are Reciprocally Modulated in Rat Cardiomyocytes'. *Experimental & Molecular Medicine* 38 (5), 535-545

Kim, Y., Bombeck, C. A., and Billiar, T. R. (1999) 'Nitric Oxide as a Bifunctional Regulator of Apoptosis'. *Circulation Research* 84 (3), 253-256

King, A. (2010) 'Cyclosporine After MI'. *Nature Reviews.Cardiology* 7 (6), 302

Kinoshita, A., Yamada, H., Kotaki, H., and Kimura, M. (2010) 'Effects of Anti-Malarial Drugs on the Electrocardiographic QT Interval Modelled in the Isolated Perfused Guinea Pig Heart System'. *Malaria Journal* 9, 318-2875-9-318

Kjekshus, J., Pedersen, T. R., Olsson, A. G., Færgeman, O., and Pyörälä, K. (1997) 'The Effects of Simvastatin on the Incidence of Heart Failure in Patients with Coronary Heart Disease'. *Journal of Cardiac Failure* 3 (4), 249-254

Klamer, D., Engel, J. A., and Svensson, L. (2001) 'The Nitric Oxide Synthase Inhibitor, L-NAME, Block Phencyclidine-Induced Disruption of Prepulse Inhibition in Mice'. *Psychopharmacology* 156 (2-3), 182-186

Kloner, R. A. and Rezkalla, S. H. (2004) 'Cardiac Protection during Acute Myocardial Infarction: Where do we Stand in 2004?'. *Journal of the American College of Cardiology* 44 (2), 276-286

Kondkar, A. A. and Abu-Amero, K. K. (2015) 'Utility of Circulating microRNAs as Clinical Biomarkers for Cardiovascular Diseases'. *BioMed Research International* 2015, 821823

Kong, R., Jia, G., Cheng, Z. X., Wang, Y. W., Mu, M., Wang, S. J., Pan, S. H., Gao, Y., Jiang, H. C., Dong, D. L., and Sun, B. (2012) 'Dihydroartemisinin Enhances Apo2L/TRAIL-Mediated Apoptosis in Pancreatic Cancer Cells Via ROS-Mediated Up-Regulation of Death Receptor 5'. *PloS One* 7 (5), e37222

Konkimalla, V. B., Blunder, M., Korn, B., Soomro, S. A., Jansen, H., Chang, W., Posner, G. H., Bauer, R., and Efferth, T. (2008) 'Effect of Artemisinins and Other Endoperoxides on Nitric Oxide-Related Signaling Pathway in RAW 264.7 Mouse Macrophage Cells'. *Nitric Oxide: Biology and Chemistry / Official Journal of the Nitric Oxide Society* 19 (2), 184-191

Krishna, S., Bustamante, L., Haynes, R. K., and Staines, H. M. (2008) 'Artemisinins: Their Growing Importance in Medicine'. *Trends in Pharmacological Sciences* 29 (10), 520-527

Krishna, S., Woodrow, C. J., Staines, H. M., Haynes, R. K., and Mercereau-Puijalon, O. (2006) 'Re-Evaluation of how Artemisinins Work in Light of Emerging Evidence of in Vitro Resistance'. *Trends in Molecular Medicine* 12 (5), 200-205

Kubes, P. and Granger, D. N. (1992) 'Nitric Oxide Modulates Microvascular Permeability'. *The American Journal of Physiology* 262 (2 Pt 2), H611-5

Kukreja, R. C., Yin, C., and Salloum, F. N. (2011) 'MicroRNAs: New Players in Cardiac Injury and Protection'. *Molecular Pharmacology* 80 (4), 558-564

Kukreja, R. C. and Xi, L. (2007) 'ENOS Phosphorylation: A Pivotal Molecular Switch in Vasodilation and Cardioprotection?'. *Journal of Molecular and Cellular Cardiology* 42 (2), 280-282

Kunapuli, S., Rosanio, S., and Schwarz, E. R. (2006) '"how do Cardiomyocytes Die?" Apoptosis and Autophagic Cell Death in Cardiac Myocytes'. *Journal of Cardiac Failure* 12 (5), 381-391

Lai, H., Sasaki, T., and Singh, N. P. (2005) 'Targeted Treatment of Cancer with Artemisinin and Artemisinin-Tagged Iron-Carrying Compounds'. *Expert Opinion on Therapeutic Targets* 9 (5), 995-1007

Lai, H. C., Singh, N. P., and Sasaki, T. (2013) 'Development of Artemisinin Compounds for Cancer Treatment'. *Investigational New Drugs* 31 (1), 230-246

Lal, S., Mahajan, A., Chen, W. N., and Chowbay, B. (2010) 'Pharmacogenetics of Target Genes Across Doxorubicin Disposition Pathway: A Review'. *Current Drug Metabolism* 11 (1), 115-128

Lala, A. and Desai, A. S. (2014) 'The Role of Coronary Artery Disease in Heart Failure'. *Heart Failure Clinics* 10 (2), 353-365

Lamberts, R. R., Hamdani, N., Soekhoe, T. W., Boontje, N. M., Zaremba, R., Walker, L. A., de Tombe, P. P., van der Velden, J., and Stienen, G. J. M. (2007) 'Frequency-Dependent Myofilament Ca2+ Desensitization in Failing Rat Myocardium'. *The Journal of Physiology* 582 (2), 695-709

Lambert, J. P., Nicholson, C. K., Amin, H., Amin, S., and Calvert, J. W. (2014) 'Hydrogen Sulfide Provides Cardioprotection Against myocardial/ischaemia Reperfusion Injury in the Diabetic State through the Activation of the RISK Pathway'. *Medical Gas Research* 4 (1), 20-014-0020-0. eCollection 2014

Lamerato, L., Havstad, S., Gandhi, S., Jones, D., and Nathanson, D. (2006) 'Economic Burden Associated with Breast Cancer Recurrence: Findings from a Retrospective Analysis of Health System Data'. *Cancer* 106 (9), 1875-1882

Layland, J., Young, I. S., and Altringham, J. D. (1997) 'The Effects of Adrenaline on the Work- and Power-Generating Capacity of Rat Papillary Muscle in Vitro'. *The Journal of Experimental Biology* 200 (Pt 3), 503-509

Layland, J., Young, I. S., and Altringham, J. D. (1995) 'The Length Dependence of Work Production in Rat Papillary Muscles in Vitro'. *The Journal of Experimental Biology* 198 (Pt 12), 2491-2499

Lee, K. L., Woodlief, L. H., Topol, E. J., Weaver, W. D., Betriu, A., Col, J., Simoons, M., Aylward, P., Van de Werf, F., and Califf, R. M. (1995) 'Predictors of 30-Day Mortality in the

Era of Reperfusion for Acute Myocardial Infarction. Results from an International Trial of 41,021 Patients. GUSTO-I Investigators'. *Circulation* 91 (6), 1659-1668

Lee, Y. and Gustafsson, A. B. (2009) 'Role of Apoptosis in Cardiovascular Disease'. *Apoptosis: An International Journal on Programmed Cell Death* 14 (4), 536-548

Le Lamer, S., Paradis, S., Rahmouni, H., Chaimbault, C., Michaud, M., Culcasi, M., Afxantidis, J., Latreille, M., Berna, P., Berdeaux, A., Pietri, S., Morin, D., Donazzolo, Y., Abitbol, J. L., Pruss, R. M., and Schaller, S. (2014) 'Translation of TRO40303 from Myocardial Infarction Models to Demonstration of Safety and Tolerance in a Randomized Phase I Trial'. *Journal of Translational Medicine* 12, 38-5876-12-38

Li, H., Cui, H., Liu, X., and Zweier, J. L. (2005) 'Xanthine Oxidase Catalyzes Anaerobic Transformation of Organic Nitrates to Nitric Oxide and Nitrosothiols: Characterization of this Mechanism and the Link between Organic Nitrate and Guanylyl Cyclase Activation'. *The Journal of Biological Chemistry* 280 (17), 16594-16600

Li, J., Levick, S. P., DiPette, D. J., Janicki, J. S., and Supowit, S. C. (2013) 'Alpha-Calcitonin Gene-Related Peptide is Protective Against Pressure Overload-Induced Heart Failure'. *Regulatory Peptides* 185, 20-28

Li, J., Xuan, W., Yan, R., Tropak, M. B., Jean-St-Michel, E., Liang, W., Gladstone, R., Backx, P. H., Kharbanda, R. K., and Redington, A. N. (2011) 'Remote Preconditioning Provides Potent Cardioprotection Via PI3K/Akt Activation and is Associated with Nuclear Accumulation of Beta-Catenin'. *Clinical Science (London, England: 1979)* 120 (10), 451-462

Li, J. and Zhou, B. (2010) 'Biological Actions of Artemisinin: Insights from Medicinal Chemistry Studies'. *Molecules (Basel, Switzerland)* 15 (3), 1378-1397

Li, L. N., Zhang, H. D., Yuan, S. J., Yang, D. X., Wang, L., and Sun, Z. X. (2008) 'Differential Sensitivity of Colorectal Cancer Cell Lines to Artesunate is Associated with Expression of Beta-Catenin and E-Cadherin'. *European Journal of Pharmacology* 588 (1), 1-8

Li, L., Cai, W., Ye, Q., Liu, J., Li, X., and Liao, X. (2014). ‘Comparison of plasma microRNA-1 and cardiac troponin T in early diagnosis of patients with acute myocardial infarction’. *World Journal of Emergency Medicine*, *5*(3), 182–186. doi:10.5847/wjem.j.issn.1920-8642.2014.03.004

Li, M. and Zhang, J. (2015) 'Circulating MicroRNAs: Potential and Emerging Biomarkers for Diagnosis of Cardiovascular and Cerebrovascular Diseases'. *BioMed Research International* 2015, 730535

Li, Y.-Q., Zhang, M.F., Wen, H.Y., Hu, C.L., Liu, R., Wei, H.Y., Li, X. (2013). ‘Comparing the diagnostic values of circulating microRNAs and cardiac troponin T in patients with acute myocardial infarction’. *Clinics*, *68*(1), 75–80. doi:10.6061/clinics/2013 (01) OA12

Libby, P. (2002) 'Inflammation in Atherosclerosis'. *Nature* 420 (6917), 868-874

Libby, P. and Theroux, P. (2005) 'Pathophysiology of Coronary Artery Disease'. *Circulation* 111 (25), 3481-3488

Lichtig, C. and Brooks, H. (1975) 'Myocardial Ultrastructure and Function during Progressive Early Ischaemia in the Intact Heart'. *The Journal of Thoracic and Cardiovascular Surgery* 70 (2), 309-315

Lieberthal, W., Tang, M., Zhang, L., Viollet, B., Patel, V., and Levine, J. S. (2013) 'Susceptibility to ATP Depletion of Primary Proximal Tubular Cell Cultures Derived from Mice Lacking either the alpha1 Or the alpha2 Isoform of the Catalytic Domain of AMPK'. *BMC Nephrology* 14, 251-2369-14-251

Lieberthal, W., Menza, S. A., and Levine, J. S. (1998) 'Graded ATP Depletion can Cause Necrosis Or Apoptosis of Cultured Mouse Proximal Tubular Cells'. *The American Journal of Physiology* 274 (2 Pt 2), F315-27

Lipinski, P., Drapier, J. -, Oliveira, L., Retmanska, H., Sochanowicz, B., and Kruszewski, M. (2000) 'Intracellular Iron Status as a Hallmark of Mammalian Cell Susceptibility to

Oxidative Stress: A Study of L5178Y Mouse Lymphoma Cell Lines Differentially Sensitive to H2O2'. *Blood* 95 (9), 2960-2966

Lipshultz, S. E., Scully, R. E., Lipsitz, S. R., Sallan, S. E., Silverman, L. B., Miller, T. L., Barry, E. V., Asselin, B. L., Athale, U., Clavell, L. A., Larsen, E., Moghrabi, A., Samson, Y., Michon, B., Schorin, M. A., Cohen, H. J., Neuberg, D. S., Orav, E. J., and Colan, S. D. (2010) 'Assessment of Dexrazoxane as a Cardioprotectant in Doxorubicin-Treated Children with High-Risk Acute Lymphoblastic Leukaemia: Long-Term Follow-Up of a Prospective, Randomised, Multicentre Trial'. *The Lancet Oncology* 11 (10), 950-961

Liu, F., Lang, J., Li, J., Benashski, S. E., Siegel, M., Xu, Y., and McCullough, L. D. (2011) 'Sex Differences in the Response to Poly (ADP-Ribose) Polymerase-1 Deletion and Caspase Inhibition After Stroke'. *Stroke; a Journal of Cerebral Circulation* 42 (4), 1090-1096

Liu, P. P., Liao, J., Tang, Z. J., Wu, W. J., Yang, J., Zeng, Z. L., Hu, Y., Wang, P., Ju, H. Q., Xu, R. H., and Huang, P. (2014) 'Metabolic Regulation of Cancer Cell Side Population by Glucose through Activation of the Akt Pathway'. *Cell Death and Differentiation* 21 (1), 124-135

Liu Z, Kastis G. A, Stevenson GD, et al. (2002) ‘Quantitative analysis of acute myocardial infarct in rat hearts with ischaemia-reperfusion using a high-resolution stationarySPECT system’. J Nucl Med. 43:933–9

Liu, T., Tang, H., Lang, Y., Liu, M., and Li, X. (2009) 'MicroRNA-27a Functions as an Oncogene in Gastric Adenocarcinoma by Targeting Prohibitin'. *Cancer Letters* 273 (2), 233-242

Liu, X., Jutooru, I., Lei, P., Kim, K., Lee, S. O., Brents, L. K., Prather, P. L., and Safe, S. (2012) 'Betulinic Acid Targets YY1 and ErbB2 through Cannabinoid Receptor-Dependent Disruption of microRNA-27a:ZBTB10 in Breast Cancer'. *Molecular Cancer Therapeutics* 11 (7), 1421-1431

Llacuna, L., Mari, M., Lluis, J. M., Garcia-Ruiz, C., Fernandez-Checa, J. C., and Morales, A. (2009) 'Reactive Oxygen Species Mediate Liver Injury through Parenchymal Nuclear

Factor-kappaB Inactivation in Prolonged ischemia/reperfusion'. *The American Journal of Pathology* 174 (5), 1776-1785

Lopez-Neblina, F., Toledo, A. H., and Toledo-Pereyra, L. (2005) 'Molecular Biology of Apoptosis in Ischaemia and Reperfusion'. *J Invest Surg* 18 (6), 335-350

Lu, Y. and Chen, G. Q. (2011) 'Effector Caspases and Leukemia'. *International Journal of Cell Biology* 2011, 738301

Luo, J., Zhu, W., Tang, Y., Cao, H., Zhou, Y., Ji, R., Zhou, X., Lu, Z., Yang, H., Zhang, S., and Cao, J. (2014) 'Artemisinin Derivative Artesunate Induces Radiosensitivity in Cervical Cancer Cells in Vitro and in Vivo'. *Radiation Oncology (London, England)* 9, 84-717X-9-84

Luo, J., Zhu, W., Tang, Y., Cao, H., Zhou, Y., Ji, R., Zhou, X., Lu, Z., Yang, H., Zhang, S., and Cao, J. (2014) 'Artemisinin Derivative Artesunate Induces Radiosensitivity in Cervical Cancer Cells in Vitro and in Vivo'. *Radiation Oncology (London, England)* 9, 84-717X-9-84

Ma, Y., Zhang, M., Ma, S., Wang, Y., Gao, J., Wang, H., and Yu, X. (2011) 'New Idea of Treatment of Heart Failure with Traditional Chinese Medical'. *Zhongguo Zhong Yao Za Zhi = Zhongguo Zhongyao Zazhi = China Journal of Chinese Materia Medica* 36 (22), 3210-3212

Ma, Y., Yu, S., Zhao, W., Lu, Z., and Chen, J. (2010) 'MiR-27a Regulates the Growth, Colony Formation and Migration of Pancreatic Cancer Cells by Targeting Sprouty2'. *Cancer Letters* 298 (2), 150-158

Ma, F. X., Zhou, B., Chen, Z., Ren, Q., Lu, S. H., Sawamura, T., and Han, Z. C. (2006) 'Oxidized Low Density Lipoprotein Impairs Endothelial Progenitor Cells by Regulation of Endothelial Nitric Oxide Synthase'. *Journal of Lipid Research* 47 (6), 1227-1237

Mackey, J. R., Clemons, M., Cote, M. A., Delgado, D., Dent, S., Paterson, A., Provencher, L., Sawyer, M. B., and Verma, S. (2008) 'Cardiac Management during Adjuvant

Trastuzumab Therapy: Recommendations of the Canadian Trastuzumab Working Group'. *Current Oncology (Toronto, Ont.)* 15 (1), 24-35

Maddika, S., Bay, G. H., Kroczak, T. J., Ande, S. R., Maddika, S., Wiechec, E., Gibson, S. B., and Los, M. (2007) 'Akt is Transferred to the Nucleus of Cells Treated with Apoptin, and it Participates in Apoptin-Induced Cell Death'. *Cell Proliferation* 40 (6), 835-848

Maddock, H. L., Mocanu, M. M., and Yellon, D. M. (2002) 'Adenosine A (3) Receptor Activation Protects the Myocardium from reperfusion/reoxygenation Injury'. *American Journal of Physiology.Heart and Circulatory Physiology*283 (4), H1307-13

Madrid, L. V., Wang, C. Y., Guttridge, D. C., Schottelius, A. J., Baldwin, A. S.,Jr, and Mayo, M. W. (2000) 'Akt Suppresses Apoptosis by Stimulating the Transactivation Potential of the RelA/p65 Subunit of NF-kappaB'. *Molecular and Cellular Biology* 20 (5), 1626-1638

Maganti, K., Rigolin, V. H., Sarano, M. E., and Bonow, R. O. (2010) 'Valvular Heart Disease: Diagnosis and Management'. *Mayo Clinic Proceedings* 85 (5), 483-500

Mahmood, T. and Yang, P. C. (2012) 'Western Blot: Technique, Theory, and Trouble Shooting'. *North American Journal of Medical Sciences* 4 (9), 429-434

Maiuri, M. C., Zalckvar, E., Kimchi, A., and Kroemer, G. (2007) 'Self-Eating and Self-Killing: Crosstalk between Autophagy and Apoptosis'. *Nature Reviews.Molecular Cell Biology* 8 (9), 741-752

Mangi, A. A., Noiseux, N., Kong, D., He, H., Rezvani, M., Ingwall, J. S., and Dzau, V. J. (2003) 'Mesenchymal Stem Cells Modified with Akt Prevent Remodeling and Restore Performance of Infarcted Hearts'. *Nature Medicine* 9 (9), 1195-1201

Massion, P. B., Feron, O., Dessy, C., and Balligand, J. -. (2003) 'Nitric Oxide and Cardiac Function: Ten Years After, and Continuing'. *Circulation Research* 93 (5), 388-398

Masters, S. C., Subramanian, R. R., Truong, A., Yang, H., Fujii, K., Zhang, H., and Fu, H. (2002) 'Survival-Promoting Functions of 14-3-3 Proteins'. *Biochemical Society Transactions* 30 (4), 360-365

Matsui, T., Tao, J., del Monte, F., Lee, K., Li, L., Picard, M., Force, T. L., Franke, T. F., Hajjar, R. J., and Rosenzweig, A. (2001) 'Akt Activation Preserves Cardiac Function and Prevents Injury After Transient Cardiac Ischaemia in Vivo'. *Circulation* 104 (3), 330-335

Maude, R. J., Woodrow, C. J., and White, L. J. (2010) 'Artemisinin Antimalarials: Preserving the "Magic Bullet"'. *Drug Development Research* 71 (1), 12-19

Maulik, N., Engelman, D. T., Watanabe, M., Engelman, R. M., Maulik, G., Cordis, G. A., and Das, D. K. (1995) 'Nitric Oxide Signaling in Ischemic Heart'. *Cardiovascular Research* 30 (4), 593-601

Mehic, B. (2012) 'Apoptosis - is it Good Or Bad'. Bosnian Journal of Basic Medical Sciences / Udruzenje Basicnih Mediciniskih Znanosti = Association of Basic Medical Sciences 12 (3), 1

Mehta, R. H., Granger, C. B., Alexander, K. P., Bossone, E., White, H. D., and Sketch Jr, M. H. (2005) 'Reperfusion Strategies for Acute Myocardial Infarction in the Elderly: Benefits and Risks'. *Journal of the American College of Cardiology* 45 (4), 471-478

Mei, Y., Yong, J., Liu, H., Shi, Y., Meinkoth, J., Dreyfuss, G., and Yang, X. (2010) 'TRNA Binds to Cytochrome c and Inhibits Caspase Activation'. *Molecular Cell* 37 (5), 668-678

Melendez, V., Peggins, J. O., Brewer, T. G., and Theoharides, A. D. (1991) 'Determination of the Antimalarial Arteether and its Deethylated Metabolite Dihydroartemisinin in Plasma by High-Performance Liquid Chromatography with Reductive Electrochemical Detection'. *Journal of Pharmaceutical Sciences* 80 (2), 132-138

Melendez, V., Peggins, J. O., Brewer, T. G., and Theoharides, A. D. (1991) 'Determination of the Antimalarial Arteether and its Deethylated Metabolite Dihydroartemisinin in Plasma by High-Performance Liquid Chromatography with Reductive Electrochemical Detection'. *Journal of Pharmaceutical Sciences* 80 (2), 132-138

Mellors, L. J. and Barclay, C. J. (2001) 'The Energetics of Rat Papillary Muscles Undergoing Realistic Strain Patterns'. *The Journal of Experimental Biology* 204 (Pt 21), 3765-3777

Mendoza, M. C., Er, E. E., and Blenis, J. (2011) 'The Ras-ERK and PI3K-mTOR Pathways: Cross-Talk and Compensation'. *Trends in Biochemical Sciences* 36 (6), 320-328Mendoza, M. C., Er, E. E., and Blenis, J. (2011) 'The Ras-ERK and PI3K-mTOR Pathways: Cross-Talk and Compensation'. *Trends in Biochemical Sciences* 36 (6), 320-328

Mercer, A. E., Copple, I. M., Maggs, J. L., O'Neill, P. M., and Park, B. K. (2011) 'The Role of Heme and the Mitochondrion in the Chemical and Molecular Mechanisms of Mammalian Cell Death Induced by the Artemisinin Antimalarials'. *The Journal of Biological Chemistry* 286 (2), 987-996

Mertens-Talcott, S. U., Chintharlapalli, S., Li, X., and Safe, S. (2007) 'The Oncogenic microRNA-27a Targets Genes that Regulate Specificity Protein Transcription Factors and the G2-M Checkpoint in MDA-MB-231 Breast Cancer Cells'. *Cancer Research* 67 (22), 11001-11011

Meshnick, S. R. (2002) 'Artemisinin: Mechanisms of Action, Resistance and Toxicity'. *International Journal for Parasitology* 32 (13), 1655-1660

Minotti, G., Menna, P., Salvatorelli, E., Cairo, G., and Gianni, L. (2004) 'Anthracyclines: Molecular Advances and Pharmacologic Developments in Antitumor Activity and Cardiotoxicity'. *Pharmacological Reviews* 56 (2), 185-229

Minotti, G., Ronchi, R., Salvatorelli, E., Menna, P., and Cairo, G. (2001) 'Doxorubicin Irreversibly Inactivates Iron Regulatory Proteins 1 and 2 in Cardiomyocytes: Evidence for Distinct Metabolic Pathways and Implications for Iron-Mediated Cardiotoxicity of Antitumor Therapy'. *Cancer Research* 61 (23), 8422-8428

Mirshafiey, A., Saadat, F., Attar, M., Di Paola, R., Sedaghat, R., and Cuzzocrea, S. (2006) 'Design of a New Line in Treatment of Experimental Rheumatoid Arthritis by Artesunate'. *Immunopharmacology and Immunotoxicology* 28 (3), 397-410

Mishina, Y. V., Krishna, S., Haynes, R. K., and Meade, J. C. (2007) 'Artemisinins Inhibit Trypanosoma Cruzi and Trypanosoma Brucei Rhodesiense in Vitro Growth'. *Antimicrobial Agents and Chemotherapy* 51 (5), 1852-1854

Miura, T. and Miki, T. (2008) 'Limitation of Myocardial Infarct Size in the Clinical Setting: Current Status and Challenges in Translating Animal Experiments into Clinical Therapy'. *Basic Research in Cardiology* 103 (6), 501-513

Mizushima, N. (2007) 'Autophagy: Process and Function'. *Genes & Development* 21 (22), 2861-2873

Minami, T., Onaka, M., Okuda-Ashitaka, E., Mori, H., Ito, S., and Hayaishi, O. (1995) 'L-NAME, an Inhibitor of Nitric Oxide Synthase, Blocks the Established Allodynia Induced by Intrathecal Administration of Prostaglandin E2'. *Neuroscience Letters* 201 (3), 239-242

Mocanu, M. M., Bell, R. M., and Yellon, D. M. (2002) 'PI3 Kinase and Not p42/p44 Appears to be Implicated in the Protection Conferred by Ischemic Preconditioning'. *Journal of Molecular and Cellular Cardiology* 34 (6), 661-668

Mocanu, M. M., Baxter, G. F., and Yellon, D. M. (2000) 'Caspase Inhibition and Limitation of Myocardial Infarct Size: Protection Against Lethal Reperfusion Injury'. *British Journal of Pharmacology* 130 (2), 197-200

Mocarski ES Jr, Shenk T, Pass RF. Cytomegalovirus.In: Fields BN, Knipe DM, Howley P M, editors. Fields virology. 5th ed. Philadelphia: Lippincott-Raven; 2007. p. 2701-72

Monassier, J. P. (2008) 'Reperfusion Injury in Acute Myocardial Infarction. from Bench to Cath Lab. Part I: Basic Considerations'. *Archives of Cardiovascular Diseases* 101 (7–8), 491-500

Moncada, S. and Higgs, A. (1993) 'The L-Arginine-Nitric Oxide Pathway'. *N Engl J Med* 329 (27), 2002-2012

Montaigne, D., Hurt, C., and Neviere, R. (2012) 'Mitochondria death/survival Signaling Pathways in Cardiotoxicity Induced by Anthracyclines and Anticancer-Targeted Therapies'. *Biochemistry Research International* 2012, 951539

Morris, S. D. and Yellon, D. M. (1997) 'Angiotensin-Converting Enzyme Inhibitors Potentiate Preconditioning through Bradykinin B2 Receptor Activation in Human Heart'. *Journal of the American College of Cardiology* 29 (7), 1599-1606

Morin, D., Assaly, R., Paradis, S., and Berdeaux, A. (2009) 'Inhibition of Mitochondrial Membrane Permeability as a Putative Pharmacological Target for Cardioprotection'. *Current Medicinal Chemistry* 16 (33), 4382-4398

Muhindo Mavoko, H., Nabasumba, C., Tinto, H., D'Alessandro, U., Grobusch, M. P., Lutumba, P., and Van Geertruyden, J. P. (2013) 'Impact of Retreatment with an Artemisinin-Based Combination on Malaria Incidence and its Potential Selection of Resistant Strains: Study Protocol for a Randomized Controlled Clinical Trial'. *Trials* 14, 307-6215-14-307

Muhindo Mavoko, H., Nabasumba, C., Tinto, H., D'Alessandro, U., Grobusch, M. P., Lutumba, P., and Van Geertruyden, J. P. (2013) 'Impact of Retreatment with an Artemisinin-Based Combination on Malaria Incidence and its Potential Selection of Resistant Strains: Study Protocol for a Randomized Controlled Clinical Trial'. *Trials* 14, 307-6215-14-307

Mukhopadhyay, P., Batkai, S., Rajesh, M., Czifra, N., Harvey-White, J., Hasko, G., Zsengeller, Z., Gerard, N. P., Liaudet, L., Kunos, G., and Pacher, P. (2007) 'Pharmacological Inhibition of CB1 Cannabinoid Receptor Protects Against Doxorubicin-Induced Cardiotoxicity'. *Journal of the American College of Cardiology* 50 (6), 528-536

Münzel T, Gori T (2013). Nitrate therapy and nitrate tolerance in patients with coronary artery disease. *Curr Opin Pharmacol* **13**:251–259

Muregi, F. W. and Ishih, A. (2010) 'Next-Generation Antimalarial Drugs: Hybrid Molecules as a New Strategy in Drug Design'. *Drug Development Research* 71 (1), 20-32

Murphy, E. and Steenbergen, C. (2008) 'Mechanisms Underlying Acute Protection from Cardiac Ischaemia-Reperfusion Injury'. *Physiological Reviews* 88 (2), 581-609

Murphy, E., Imahashi, K., and Steenbergen, C. (2005) 'Bcl-2 Regulation of Mitochondrial Energetics'. *Trends in Cardiovascular Medicine* 15 (8), 283-290

Murray, C. J. and Lopez, A. D. (1997) 'Alternative Projections of Mortality and Disability by Cause 1990-2020: Global Burden of Disease Study'. *Lancet (London, England)* 349 (9064), 1498-1504

Mwai, L., Ochong, E., Abdirahman, A., Kiara, S. M., Ward, S., Kokwaro, G., Sasi, P., Marsh, K., Borrmann, S., Mackinnon, M., and Nzila, A. (2009) 'Chloroquine Resistance before and After its Withdrawal in Kenya'. *Malaria Journal* 8, 106-2875-8-106

Nakase, I., Gallis, B., Takatani-Nakase, T., Oh, S., Lacoste, E., Singh, N. P., Goodlett, D. R., Tanaka, S., Futaki, S., Lai, H., and Sasaki, T. (2009) 'Transferrin Receptor-Dependent Cytotoxicity of Artemisinin-Transferrin Conjugates on Prostate Cancer Cells and Induction of Apoptosis'. *Cancer Letters* 274 (2), 290-298

Nakase, I., Lai, H., Singh, N. P., and Sasaki, T. (2008) 'Anticancer Properties of Artemisinin Derivatives and their Targeted Delivery by Transferrin Conjugation'. *International Journal of Pharmaceutics* 354 (1-2), 28-33

Nam, W., Tak, J., Ryu, J. K., Jung, M., Yook, J. I., Kim, H. J., and Cha, I. H. (2007) 'Effects of Artemisinin and its Derivatives on Growth Inhibition and Apoptosis of Oral Cancer Cells'. *Head & Neck* 29 (4), 335-340

Nazari-Jahantigh, M., Wei, Y., Noels, H., Akhtar, S., Zhou, Z., Koenen, R. R., Heyll, K., Gremse, F., Kiessling, F., Grommes, J., Weber, C., and Schober, A. (2012) 'MicroRNA-155 Promotes Atherosclerosis by Repressing Bcl6 in Macrophages'. *The Journal of Clinical Investigation* 122 (11), 4190-4202

Ng, R., Better, N., and Green, M. D. (2006) 'Anticancer Agents and Cardiotoxicity'. *Seminars in Oncology* 33 (1), 2-14

Ng, S. S., Tsao, M. S., Nicklee, T., and Hedley, D. W. (2001) 'Wortmannin Inhibits pkb/akt Phosphorylation and Promotes Gemcitabine Antitumor Activity in Orthotopic Human Pancreatic Cancer Xenografts in Immunodeficient Mice'. *Clinical Cancer Research: An Official Journal of the American Association for Cancer Research* 7 (10), 3269-3275

NHS (2014). ‘Coronary heart disease’. Available online from < http://www.nhs.uk/conditions/Coronary-heart-disease/Pages/Introduction.aspx>.

Niccoli, G., Scalone, G., and Crea, F. (2014) 'Acute Myocardial Infarction with no Obstructive Coronary Atherosclerosis: Mechanisms and Management'. *European Heart Journal*

Nichols, M., Townsend, N., Scarborough, P., and Rayner, M. (2014) 'Cardiovascular Disease in Europe 2014: Epidemiological Update'. *European Heart Journal* 35 (42), 2950-2959

Nicholson, D. W., Ali, A., Thornberry, N. A., Vaillancourt, J. P., Ding, C. K., Gallant, M., Gareau, Y., Griffin, P. R., Labelle, M., and Lazebnik, Y. A. (1995) 'Identification and Inhibition of the ICE/CED-3 Protease Necessary for Mammalian Apoptosis'. *Nature* 376 (6535), 37-43

Nishi, H., Ono, K., Horie, T., Nagao, K., Kinoshita, M., Kuwabara, Y., Watanabe, S., Takaya, T., Tamaki, Y., Takanabe-Mori, R., Wada, H., Hasegawa, K., Iwanaga, Y., Kawamura, T., Kita, T., and Kimura, T. (2011) 'MicroRNA-27a Regulates Beta Cardiac Myosin Heavy Chain Gene Expression by Targeting Thyroid Hormone Receptor beta1 in Neonatal Rat Ventricular Myocytes'. *Molecular and Cellular Biology* 31 (4), 744-755

NIH (2011). ‘What is Angina’. Available onine from< https://www.nhlbi.nih.gov/health/health-topics/topics/angina/>

NIH (2014). ‘Atherosclerosis’. Available online from <http://www.nhlbi.nih.gov/health/health- topics/topics/atherosclerosis>

Nohria, A. (2013) 'ß-Adrenergic Blockade for Anthracycline- and Trastuzumab-Induced Cardiotoxicity: Is Prevention Better than Cure?'. *Circulation: Heart Failure* 6 (3), 358-361

Nosten, F. and White, N. J. (2007) 'Artemisinin-Based Combination Treatment of Falciparum Malaria'. *The American Journal of Tropical Medicine and Hygiene* 77 (6 Suppl), 181-192

Nosten, F. and White, N. J. (2007) 'Artemisinin-Based Combination Treatment of Falciparum Malaria'. *The American Journal of Tropical Medicine and Hygiene* 77 (6 Suppl), 181-192

NSBBio (2010). Available online from<http://www.nbsbio.co.uk/img/productImages/MTT_Principle.JPG>

Octavia, Y., Tocchetti, C. G., Gabrielson, K. L., Janssens, S., Crijns, H. J., and Moens, A. L. (2012) 'Doxorubicin-Induced Cardiomyopathy: From Molecular Mechanisms to Therapeutic Strategies'. *Journal of Molecular and Cellular Cardiology* 52 (6), 1213-1225

Oh, S., Kim, B. J., Singh, N. P., Lai, H., and Sasaki, T. (2009) 'Synthesis and Anti-Cancer Activity of Covalent Conjugates of Artemisinin and a Transferrin-Receptor Targeting Peptide'. *Cancer Letters* 274 (1), 33-39

Okada, H. and Mak, T. W. (2004) 'Pathways of Apoptotic and Non-Apoptotic Death in Tumour Cells'. *Nature Reviews.Cancer* 4 (8), 592-603

Olle, E. W., Sreekumar, A., Warner, R. L., McClintock, S. D., Chinnaiyan, A. M., Bleavins, M. R., Anderson, T. D., and Johnson, K. J. (2005) 'Development of an Internally Controlled Antibody Microarray'. *Molecular & Cellular Proteomics: MCP* 4 (11), 1664-1672

Olson, E. N. (2014) 'MicroRNAs as Therapeutic Targets and Biomarkers of Cardiovascular Disease'. *Science Translational Medicine* 6 (239), 239ps3-239ps3

Ong, S. B., Dongworth, R. K., Cabrera-Fuentes, H. A., and Hausenloy, D. J. (2015) 'Role of the MPTP in Conditioning the Heart - Translatability and Mechanism'. *British Journal of Pharmacology* 172 (8), 2074-2084

O'Neill, P. M., Barton, V. E., and Ward, S. A. (2010) 'The Molecular Mechanism of Action of Artemisinin--the Debate Continues'. *Molecules (Basel, Switzerland)* 15 (3), 1705-1721

O'Neill, P. M. and Posner, G. H. (2004) 'A Medicinal Chemistry Perspective on Artemisinin and Related Endoperoxides'. *Journal of Medicinal Chemistry* 47 (12), 2945-2964

Oudit, G. Y. and Penninger, J. M. (2009) 'Cardiac Regulation by Phosphoinositide 3-Kinases and PTEN'. *Cardiovascular Research* 82 (2), 250-260

Ozaki, M., Kawashima, S., Hirase, T., Yamashita, T., Namiki, M., Inoue, N., Hirata Ki, K., and Yokoyama, M. (2002) 'Overexpression of Endothelial Nitric Oxide Synthase in Endothelial Cells is Protective Against Ischaemia-Reperfusion Injury in Mouse Skeletal Muscle'. *The American Journal of Pathology* 160 (4), 1335-1344

Pacher, P., Liaudet, L., Bai, P., Mabley, J. G., Kaminski, P. M., Virag, L., Deb, A., Szabo, E., Ungvari, Z., Wolin, M. S., Groves, J. T., and Szabo, C. (2003) 'Potent Metalloporphyrin Peroxynitrite Decomposition Catalyst Protects Against the Development of Doxorubicin-Induced Cardiac Dysfunction'. *Circulation* 107 (6), 896-904

Pacher, P., Beckman, J. S., and Liaudet, L. (2007) 'Nitric Oxide and Peroxynitrite in Health and Disease'. *Physiological Reviews* 87 (1), 315-424

Paik, I. H., Xie, S., Shapiro, T. A., Labonte, T., Narducci Sarjeant, A. A., Baege, A. C., and Posner, G. H. (2006) 'Second Generation, Orally Active, Antimalarial, Artemisinin-Derived Trioxane Dimers with High Stability, Efficacy, and Anticancer Activity'. *Journal of Medicinal Chemistry* 49 (9), 2731-2734

Palanca-Wessels, M. C. and Press, O. W. (2014) 'Advances in the Treatment of Hematologic Malignancies using Immunoconjugates'. *Blood* 123 (15), 2293-2301

Papageorgiou, N., Tousoulis, D., Androulakis, E., Siasos, G., Briasoulis, A., Vogiatzi, G., Kampoli, A. M., Tsiamis, E., Tentolouris, C., and Stefanadis, C. (2012) 'The Role of microRNAs in Cardiovascular Disease'. *Current Medicinal Chemistry* 19 (16), 2605-2610

Park, E. S., Kang, D. H., Yang, M. K., Kang, J. C., Jang, Y. C., Park, J. S., Kim, S. K., and Shin, H. S. (2014) 'Cordycepin, 3'-Deoxyadenosine, Prevents Rat Hearts from ischemia/reperfusion Injury Via Activation of Akt/GSK-3beta/p70S6K Signaling Pathway and HO-1 Expression'. Cardiovascular Toxicology 14 (1), 1-9

Park, C., Hong, S. H., Kim, G. Y., and Choi, Y. H. (2015) 'So-Cheong-Ryong-Tang Induces Apoptosis through Activation of the Intrinsic and Extrinsic Apoptosis Pathways, and Inhibition of the PI3K/Akt Signaling Pathway in Non-Small-Cell Lung Cancer A549 Cells'. *BMC Complementary and Alternative Medicine* 15, 113-015-0639-y

Park, E. S., Kim, S. D., Lee, M. H., Lee, H. S., Lee, I. S., Sung, J. K., and Yoon, Y. S. (2003) 'Protective Effects of N-Acetylcysteine and Selenium Against Doxorubicin Toxicity in Rats'. *Journal of Veterinary Science* 4 (2), 129-136

Parker, J. D., Parker, A. B., Farrell, B., and Parker, J. O. (1996) 'Effects of Diuretic Therapy on the Development of Tolerance to Nitroglycerin and Exercise Capacity in Patients with Chronic Stable Angina'. *Circulation* 93 (4), 691-696

Pasvol, G. (2005) 'The Treatment of Complicated and Severe Malaria'. *British Medical Bulletin* 75-76 (1), 29-47

Peake, J. and Suzuki, K. (2004) 'Neutrophil Activation, Antioxidant Supplements and Exercise-Induced Oxidative Stress'. *Exercise Immunology Review* 10, 129-141

Penna, C., Settanni, F., Tullio, F., Trovato, L., Pagliaro, P., Alloatti, G., Ghigo, E., and Granata, R. (2013) 'GH-Releasing Hormone Induces Cardioprotection in Isolated Male Rat Heart Via Activation of RISK and SAFE Pathways'. *Endocrinology* 154 (4), 1624-1635

Peters, W. P., Ross, M., Vredenburgh, J. J., Meisenberg, B., Marks, L. B., Winer, E., Kurtzberg, J., Bast, R. C.,Jr, Jones, R., and Shpall, E. (1993) 'High-Dose Chemotherapy and Autologous Bone Marrow Support as Consolidation After Standard-Dose Adjuvant Therapy for High-Risk Primary Breast Cancer'. *Journal of Clinical Oncology : Official Journal of the American Society of Clinical Oncology* 11 (6), 1132-1143

Pham-Huy, L. A., He, H., and Pham-Huy, C. (2008) 'Free Radicals, Antioxidants in Disease and Health'. *International Journal of Biomedical Science: IJBS* 4 (2), 89-96

Piper, H. M. (2000) 'The Calcium Paradox Revisited: An Artefact of Great Heuristic Value'. *Cardiovascular Research* 45 (1), 123-127

Piot, C., Croisille, P., Staat, P., Thibault, H., Rioufol, G., Mewton, N., Elbelghiti, R., Cung, T. T., Bonnefoy, E., Angoulvant, D., Macia, C., Raczka, F., Sportouch, C., Gahide, G., Finet, G., Andre-Fouet, X., Revel, D., Kirkorian, G., Monassier, J. P., Derumeaux, G., and Ovize, M. (2008) 'Effect of Cyclosporine on Reperfusion Injury in Acute Myocardial Infarction'. *The New England Journal of Medicine* 359 (5), 473-481

Poljsak, B., Suput, D., and Milisav, I. (2013) 'Achieving the Balance between ROS and Antioxidants: When to use the Synthetic Antioxidants'. *Oxidative Medicine and Cellular Longevity* 2013, 956792

Pomblum, V. J., Korbmacher, B., Cleveland, S., Sunderdiek, U., Klocke, R. C., and Schipke, J. D. (2010) 'Cardiac Stunning in the Clinic: The Full Picture'. *Interactive CardioVascular and Thoracic Surgery* 10 (1), 86-91

Poole-Wilson, P. A. (1984) 'Potassium and the Heart'. *Clinics in Endocrinology and Metabolism* 13 (2), 249-268

Poon, B. Y., Raharjo, E., Patel, K. D., Tavener, S., and Kubes, P. (2003) 'Complexity of Inducible Nitric Oxide Synthase: Cellular Source Determines Benefit Versus Toxicity'. *Circulation* 108 (9), 1107-1112

Port, J. D. and Sucharov, C. (2010) 'Role of microRNAs in Cardiovascular Disease: Therapeutic Challenges and Potentials'. *Journal of Cardiovascular Pharmacology* 56 (5), 444-453

Portt, L., Norman, G., Clapp, C., Greenwood, M., and Greenwood, M. T. (2011) 'Anti-Apoptosis and Cell Survival: A Review'. *Biochimica Et Biophysica Acta* 1813 (1), 238-259

Poulter, N. (1999) 'Coronary Heart Disease is a Multifactorial Disease'. *American Journal of Hypertension* 12 (S6), 92S-95S

Poyton, R. O., Ball, K. A., and Castello, P. R. (2009) 'Mitochondrial Generation of Free Radicals and Hypoxic Signaling'. *Trends in Endocrinology & Metabolism* 20 (7), 332-340

Praygod, G., de Frey, A., and Eisenhut, M. (2008) 'Artemisinin Derivatives Versus Quinine in Treating Severe Malaria in Children: A Systematic Review'. *Malaria Journal* 7, 210-2875-7-210

Pritsos, C. A. (2000) 'Cellular Distribution, Metabolism and Regulation of the Xanthine Oxidoreductase Enzyme System'. *Chemico-Biological Interactions* 129 (1-2), 195-208

Qaderi, A., Dadgar, N., Mansouri, H., Alavi, S. E., Esfahani, M. K., and Akbarzadeh, A. (2013) 'Modeling and Prediction of Cytotoxicity of Artemisinin for Treatment of the Breast Cancer by using Artificial Neural Networks'. *Springerplus* 2, 340-1801-2-340. eCollection 2013

Quintana, M., Hjemdahl, P., Sollevi, A., Kahan, T., Edner, M., Rehnqvist, N., Swahn, E., Kjerr, A. C., Nasman, P., and ATTACC investigators (2003) 'Left Ventricular Function and Cardiovascular Events Following Adjuvant Therapy with Adenosine in Acute Myocardial Infarction Treated with Thrombolysis, Results of the ATTenuation by Adenosine of Cardiac Complications (ATTACC) Study'. *European Journal of Clinical Pharmacology* 59 (1), 1-9

Rada, B. and Leto, T. L. (2008) 'Oxidative Innate Immune Defenses by Nox/Duox Family NADPH Oxidases'. *Contributions to Microbiology* 15, 164-187

Rahman, K. (2007) 'Studies on Free Radicals, Antioxidants, and Co-Factors'. *Clinical Interventions in Aging* 2 (2), 219-236

Raij, L. (2006) 'Nitric Oxide in the Pathogenesis of Cardiac Disease'. *Journal of Clinical Hypertension (Greenwich, Conn.)* 8 (12 Suppl 4), 30-39

Ramirez, L., Zabaleta, E. J., and Lamattina, L. (2010) 'Nitric Oxide and Frataxin: Two Players Contributing to Maintain Cellular Iron Homeostasis'. *Annals of Botany* 105 (5), 801-810

Ramos-Martin, V., Gonzalez-Martinez, C., Mackenzie, I., Schmutzhard, J., Pace, C., Lalloo, D. G., and Terlouw, D. J. (2014) 'Neuroauditory Toxicity of Artemisinin Combination

Therapies-have Safety Concerns been Addressed?'. *The American Journal of Tropical Medicine and Hygiene* 91 (1), 62-73

Raschi, E., Vasina, V., Ursino, M. G., Boriani, G., Martoni, A., and De Ponti, F. (2010) 'Anticancer Drugs and Cardiotoxicity: Insights and Perspectives in the Era of Targeted Therapy'. *Pharmacology & Therapeutics* 125 (2), 196-218

Rath, N. and Olson, M. F. (2012) 'Rho-Associated Kinases in Tumorigenesis: Re-Considering ROCK Inhibition for Cancer Therapy'. *EMBO Reports* 13 (10), 900-908

Ratovitski, E. A. (2013) 'Tumor Protein p63/microRNA Network in Epithelial Cancer Cells'. *Current Genomics* 14 (7), 441-452

Redfors, B., Shao, Y., and Omerovic, E. (2012) 'Myocardial Infarct Size and Area at Risk Assessment in Mice'. *Experimental and Clinical Cardiology* 17 (4), 268-272

Reynolds, H. R. (2012) 'Myocardial Infarction without Obstructive Coronary Artery Disease'. *Current Opinion in Cardiology* 27 (6), 655-660

Rezkalla, S. H. and Kloner, R. A. (2008) 'Coronary no-Reflow Phenomenon: From the Experimental Laboratory to the Cardiac Catheterization Laboratory'. *Catheterization and Cardiovascular Interventions : Official Journal of the Society for Cardiac Angiography & Interventions* 72 (7), 950-957

Ribichini, F. and Wijns, W. (2002) 'Acute Myocardial Infarction: Reperfusion Treatment'. *Heart* 88 (3), 298-305

Riganti, C., Doublier, S., Viarisio, D., Miraglia, E., Pescarmona, G., Ghigo, D., and Bosia, A. (2009) 'Artemisinin Induces Doxorubicin Resistance in Human Colon Cancer Cells Via Calcium-Dependent Activation of HIF-1alpha and P-Glycoprotein Overexpression'. *British Journal of Pharmacology* 156 (7), 1054-1066

Riganti, C., Miraglia, E., Viarisio, D., Costamagna, C., Pescarmona, G., Ghigo, D., and Bosia, A. (2005) 'Nitric Oxide Reverts the Resistance to Doxorubicin in Human Colon Cancer Cells by Inhibiting the Drug Efflux'. *Cancer Research* 65 (2), 516-525

Rivera, J., Sobey, C. G., Walduck, A. K., and Drummond, G. R. (2010) 'Nox Isoforms in Vascular Pathophysiology: Insights from Transgenic and Knockout Mouse Models'. *Redox Report: Communications in Free Radical Research* 15 (2), 50-63

Roberts, P. J. and Der, C. J. (2007) 'Targeting the Raf-MEK-ERK Mitogen-Activated Protein Kinase Cascade for the Treatment of Cancer'. *Oncogene* 26 (22), 3291-3310

Rodrigo, R., Libuy, M., Feliu, F., and Hasson, D. (2013) 'Molecular Basis of Cardioprotective Effect of Antioxidant Vitamins in Myocardial Infarction'. *BioMed Research International* 2013, 437613

Rodriguez-Viciana, P., Warne, P. H., Dhand, R., Vanhaesebroeck, B., Gout, I., Fry, M. J., Waterfield, M. D., and Downward, J. (1994) 'Phosphatidylinositol-3-OH Kinase Direct Target of Ras'

Roos, W. P. and Kaina, B. (2006) 'DNA Damage-Induced Cell Death by Apoptosis'. *Trends in Molecular Medicine* 12 (9), 440-450

Rosano, G. M., Fini, M., Caminiti, G., and Barbaro, G. (2008) 'Cardiac Metabolism in Myocardial Ischaemia'. *Current Pharmaceutical Design* 14 (25), 2551-2562

Ross, A. M., Gibbons, R. J., Stone, G. W., Kloner, R. A., and Alexander, R. W. (2005) 'A Randomized, Double-Blinded, Placebo-Controlled Multicenter Trial of Adenosine as an Adjunct to Reperfusion in the Treatment of Acute Myocardial Infarction (AMISTAD-II)'. *Journal of the American College of Cardiology* 45 (11), 1775-1780

Rosenfeldt, F., Wilson, M., Lee, G., Kure, C., Ou, R., Braun, L., and de Haan, J. (2013) 'Oxidative Stress in Surgery in an Ageing Population: Pathophysiology and Therapy'. *Experimental Gerontology* 48 (1), 45-54

Romond, E. H., Perez, E. A., Bryant, J., Suman, V. J., Geyer, C. E., Davidson, N. E., Tan-Chiu, E., Martino, S., Paik, S., Kaufman, P. A., Swain, S. M., Pisansky, T. M., Fehrenbacher, L., Kutteh, L. A., Vogel, V. G., Visscher, D. W., Yothers, G., Jenkins, R. B., Brown, A. M., Dakhil, S. R., Mamounas, E. P., Lingle, W. L., Klein, P. M., Ingle, J. N., and Wolmark, N.

(2005) 'Trastuzumab Plus Adjuvant Chemotherapy for Operable HER2-Positive Breast Cancer'. *N Engl J Med* 353 (16), 1673-1684

Russell, S. D., Blackwell, K. L., Lawrence, J., Pippen, J. E., Jr, Roe, M. T., Wood, F., Paton, V., Holmgren, E., and Mahaffey, K. W. (2010) 'Independent Adjudication of Symptomatic Heart Failure with the use of Doxorubicin and Cyclophosphamide Followed by Trastuzumab Adjuvant Therapy: A Combined Review of Cardiac Data from the National Surgical Adjuvant Breast and Bowel Project B-31 and the North Central Cancer Treatment Group N9831 Clinical Trials'. *Journal of Clinical Oncology: Official Journal of the American Society of Clinical Oncology* 28 (21), 3416-3421

Sakamaki, K. and Satou, Y. (2009) 'Caspases: Evolutionary Aspects of their Functions in Vertebrates'. *Journal of Fish Biology* 74 (4), 727-753

Salic, K. and De Windt, L. J. (2012) 'MicroRNAs as Biomarkers for Myocardial Infarction'. *Current Atherosclerosis Reports* 14 (3), 193-200

Salminen, A., Lehtonen, M., Suuronen, T., Kaarniranta, K., and Huuskonen, J. (2008) 'Terpenoids: Natural Inhibitors of NF-kappaB Signaling with Anti-Inflammatory and Anticancer Potential'. *Cellular and Molecular Life Sciences: CMLS* 65 (19), 2979-2999

Salloum, F., Yin, C., Xi, L., and Kukreja, R. C. (2003) 'Sildenafil Induces Delayed Preconditioning through Inducible Nitric Oxide Synthase-Dependent Pathway in Mouse Heart'. *Circulation Research* 92 (6), 595-597

Sancho, P., Fernandez, C., Yuste, V. J., Amran, D., Ramos, A. M., de Blas, E., Susin, S. A., and Aller, P. (2006) 'Regulation of apoptosis/necrosis Execution in Cadmium-Treated Human Promonocytic Cells Under Different Forms of Oxidative Stress'. *Apoptosis : An International Journal on Programmed Cell Death* 11 (5), 673-686

Sandhu, H. and Maddock, H. (2014) 'Molecular Basis of Cancer-Therapy-Induced Cardiotoxicity: Introducing microRNA Biomarkers for Early Assessment of Subclinical Myocardial Injury'. *Clinical Science (London, England : 1979)*126 (6), 377-400

Santos, F. A., Bastos, E. M., Uzeda, M., Carvalho, M. A., Farias, L. M., Moreira, E. S., and Braga, F. C. (2002) 'Antibacterial Activity of Brazilian Propolis and Fractions Against Oral Anaerobic Bacteria'. *Journal of Ethnopharmacology* 80 (1), 1-7

Sarbassov DD, Guertin DA, Ali SM, Sabatini DM. (2005) ‘Phosphorylation and regulation of Akt/PKB by the rictor-mTOR complex’. Science 307(5712):1098- 101.

Sargent, D., Sobrero, A., Grothey, A., O'Connell, M. J., Buyse, M., Andre, T., Zheng, Y., Green, E., Labianca, R., O'Callaghan, C., Seitz, J. F., Francini, G., Haller, D., Yothers, G., Goldberg, R., and de Gramont, A. (2009) 'Evidence for Cure by Adjuvant Therapy in Colon Cancer: Observations Based on Individual Patient Data from 20,898 Patients on 18 Randomized Trials'. *Journal of Clinical Oncology* 27 (6), 872-877

Satoh, M., Minami, Y., Takahashi, Y., Tabuchi, T., and Nakamura, M. (2010) 'Expression of microRNA-208 is Associated with Adverse Clinical Outcomes in Human Dilated Cardiomyopathy'. *Journal of Cardiac Failure* 16 (5), 404-410

Saiko, P., Graser, G., Giessrigl, B., Lackner, A., Grusch, M., Krupitza, G., Basu, A., Sinha, B. N., Jayaprakash, V., Jaeger, W., Fritzer-Szekeres, M., and Szekeres, T. (2011) 'A Novel N-Hydroxy-N'-Aminoguanidine Derivative Inhibits Ribonucleotide Reductase Activity: Effects in Human HL-60 Promyelocytic Leukemia Cells and Synergism with Arabinofuranosylcytosine (Ara-C)'. *Biochemical Pharmacology* 81 (1), 50-59

Schönbeck, U., Sukhova, G. K., Shimizu, K., Mach, F., and Libby, P. (2000) 'Inhibition of CD40 Signaling Limits Evolution of Established Atherosclerosis in Mice'. *Proceedings of the National Academy of Sciences* 97 (13), 7458-7463

Schroen, B. and Heymans, S. (2009) 'MicroRNAs and Beyond: The Heart Reveals its Treasures'. *Hypertension* 54 (6), 1189-1194

Schultz, N. A., Dehlendorff, C., Jensen, B. V., Bjerregaard, J. K., Nielsen, K. R., Bojesen, S. E., Calatayud, D., Nielsen, S. E., Yilmaz, M., Hollander, N. H., Andersen, K. K., and Johansen, J. S. (2014) 'MicroRNA Biomarkers in Whole Blood for Detection of Pancreatic Cancer'. *Jama* 311 (4), 392-404

Schwartz, C. L., Constine, L. S., Villaluna, D., London, W. B., Hutchison, R. E., Sposto, R., Lipshultz, S. E., Turner, C. S., deAlarcon, P. A., and Chauvenet, A. (2009) 'A Risk-Adapted, Response-Based Approach using ABVE-PC for Children and Adolescents with Intermediate- and High-Risk Hodgkin Lymphoma: The Results of P9425'. *Blood* 114 (10), 2051-2059

Searle, J., Kerr, J. F., and Bishop, C. J. (1982) 'Necrosis and Apoptosis: Distinct Modes of Cell Death with Fundamentally Different Significance'. *Pathology Annual* 17 Pt 2, 229-259

Seok, H. Y., Chen, J., Kataoka, M., Huang, Z. P., Ding, J., Yan, J., Hu, X., and Wang, D. Z. (2014) 'Loss of microRNA-155 Protects the Heart from Pathological Cardiac Hypertrophy'. *Circulation Research*

Shaw, L. J., Berman, D. S., Maron, D. J., Mancini, G. B. J., Hayes, S. W., Hartigan, P. M., Weintraub, W. S., O'Rourke, R. A., Dada, M., Spertus, J. A., Chaitman, B. R., Friedman, J., Slomka, P., Heller, G. V., Germano, G., Gosselin, G., Berger, P., Kostuk, W. J., Schwartz, R. G., Knudtson, M., Veledar, E., Bates, E. R., McCallister, B., Teo, K. K., Boden, W. E., and for the COURAGE Investigators (2008) 'Optimal Medical Therapy with Or without Percutaneous Coronary Intervention to Reduce Ischemic Burden: Results from the Clinical Outcomes Utilizing Revascularization and Aggressive Drug Evaluation (COURAGE) Trial Nuclear Substudy'. *Circulation*117 (10), 1283-1291

Sharma, A., Raghavendra, K., Adak, T., and Dash, A. P. (2008) 'Determination of Nitric Oxide Metabolites, Nitrate and Nitrite, in Anopheles Culicifacies Mosquito Midgut and Haemolymph by Anion Exchange High-Performance Liquid Chromatography: Plausible Mechanism of Refractoriness'. *Malaria Journal* 7, 71-2875-7-71

Shen, M., Ge, H. L., He, Y. X., Song, Q. L., and Zhang, H. Z. (1984) 'Immunosuppressive Action of Qinghaosu.'. *Scientia Sinica.Series B, Chemical, Biological, Agricultural, Medical & Earth Sciences / Chung-Kuo k''o Hsueh Yuan, Chu Pan* 27 (4), 398-406

Shi, J. Q., Zhang, C. C., Sun, X. L., Cheng, X. X., Wang, J. B., Zhang, Y. D., Xu, J., and Zou, H. Q. (2013) 'Antimalarial Drug Artemisinin Extenuates Amyloidogenesis and Neuroinflammation in APPswe/PS1dE9 Transgenic Mice Via Inhibition of Nuclear Factor-

kappaB and NLRP3 Inflammasome Activation'. *CNS Neuroscience & Therapeutics* 19 (4), 262-268

Shi, Y., Moon, M., Dawood, S., McManus, B., and Liu, P. P. (2011) 'Mechanisms and Management of Doxorubicin Cardiotoxicity'. *Herz* 36 (4), 296-305

Shi, J., Zhang, L., Zhang, Y. W., Surma, M., Mark Payne, R., and Wei, L. (2012) 'Downregulation of Doxorubicin-Induced Myocardial Apoptosis Accompanies Postnatal Heart Maturation'. *American Journal of Physiology.Heart and Circulatory Physiology* 302 (8), H1603-13

Shigekawa, M. and Iwamoto, T. (2001) 'Cardiac Na+-Ca2+ Exchange : Molecular and Pharmacological Aspects'. *Circulation Research* 88 (9), 864-876

Shinzawa, K. and Tsujimoto, Y. (2003) 'PLA2 Activity is Required for Nuclear Shrinkage in Caspase-Independent Cell Death'. *The Journal of Cell Biology* 163 (6), 1219-1230

Shneyvays, V., Leshem, D., Zinman, T., Mamedova, L. K., Jacobson, K. A., and Shainberg, A. (2005) 'Role of Adenosine A1 and A3 Receptors in Regulation of Cardiomyocyte Homeostasis After Mitochondrial Respiratory Chain Injury'. *American Journal of Physiology.Heart and Circulatory Physiology* 288 (6), H2792-801

Simunek, T., Sterba, M., Popelova, O., Adamcova, M., Hrdina, R., and Gersl, V. (2009) 'Anthracycline-Induced Cardiotoxicity: Overview of Studies Examining the Roles of Oxidative Stress and Free Cellular Iron'. *Pharmacological Reports : PR* 61 (1), 154-171

Simunek, T., Sterba, M., Popelova, O., Kaiserova, H., Adamcova, M., Hroch, M., Haskova, P., Ponka, P., and Gersl, V. (2008) 'Anthracycline Toxicity to Cardiomyocytes Or Cancer Cells is Differently Affected by Iron Chelation with Salicylaldehyde Isonicotinoyl Hydrazone'. *British Journal of Pharmacology* 155 (1), 138-148

Siedle, B., Garcia-Pineres, A. J., Murillo, R., Schulte-Monting, J., Castro, V., Rungeler, P., Klaas, C. A., Da Costa, F. B., Kisiel, W., and Merfort, I. (2004) 'Quantitative Structure-

Activity Relationship of Sesquiterpene Lactones as Inhibitors of the Transcription Factor NF-kappaB'. *Journal of Medicinal Chemistry* 47 (24), 6042-6054

Silachev, D. N., Plotnikov, E. Y., Pevzner, I. B., Zorova, L. D., Babenko, V. A., Zorov, S. D., Popkov, V. A., Jankauskas, S. S., Zinchenko, V. P., Sukhikh, G. T., and Zorov, D. B. (2014) 'The Mitochondrion as a Key Regulator of Ischaemic Tolerance and Injury'. *Heart, Lung and Circulation* 23 (10), 897-904

Simanek, R., Wuensch, M., Edlinger, R., Hammerl-Ferrari, B., Kramer, L., and Geissler, K. (2010) 'Comorbidity Oriented Oncology - an Overview'. *Wiener Klinische Wochenschrift* 122 (7-8), 203-218

Simon, R. (2014) 'Post-Conditioning and Reperfusion Injury in the Treatment of Stroke'. *Dose-Response: A Publication of International Hormesis Society* 12 (4), 590-599

Singh, N. P., Lai, H. C., Park, J. S., Gerhardt, T. E., Kim, B. J., Wang, S., and Sasaki, T. (2011) 'Effects of Artemisinin Dimers on Rat Breast Cancer Cells in Vitro and in Vivo'. *Anticancer Research* 31 (12), 4111-4114

Singh, N. P. and Lai, H. C. (2004) 'Artemisinin Induces Apoptosis in Human Cancer Cells'. *Anticancer Research* 24 (4), 2277-2280

Singh, N. P. and Lai, H. (2001) 'Selective Toxicity of Dihydroartemisinin and Holotransferrin Toward Human Breast Cancer Cells'. *Life Sciences* 70 (1), 49-56

Sivaraman, V. and Yellon, D. M. (2014) 'Pharmacologic Therapy that Simulates Conditioning for Cardiac Ischemic/Reperfusion Injury'. *Journal of Cardiovascular Pharmacology and Therapeutics* 19 (1), 83-96

Slamon, D. J., Leyland-Jones, B., Shak, S., Fuchs, H., Paton, V., Bajamonde, A., Fleming, T., Eiermann, W., Wolter, J., Pegram, M., Baselga, J., and Norton, L. (2001) 'Use of Chemotherapy Plus a Monoclonal Antibody Against HER2 for Metastatic Breast Cancer that Overexpresses HER2'. *N Engl J Med* 344 (11), 783-792

Sparano, J. A., Brown, D. L., and Wolff, A. C. (2002) 'Predicting Cancer Therapy-Induced Cardiotoxicity: The Role of Troponins and Other Markers'. *Drug Safety* 25 (5), 301-311

Stockert, J. C., Blázquez-Castro, A., Cañete, M., Horobin, R. W., and Villanueva, Á. (2012) 'MTT Assay for Cell Viability: Intracellular Localization of the Formazan Product is in Lipid Droplets'. *Acta Histochemica* 114 (8), 785-796

Swain, S. M., Whaley, F. S., Gerber, M. C., Weisberg, S., York, M., Spicer, D., Jones, S. E., Wadler, S., Desai, A., Vogel, C., Speyer, J., Mittelman, A., Reddy, S., Pendergrass, K., Velez-Garcia, E., Ewer, M. S., Bianchine, J. R., and Gams, R. A. (1997) 'Cardioprotection with Dexrazoxane for Doxorubicin-Containing Therapy in Advanced Breast Cancer'. *Journal of Clinical Oncology: Official Journal of the American Society of Clinical Oncology* 15 (4), 1318-1332

Skyschally, A., Schulz, R., and Heusch, G. (2008) 'Pathophysiology of Myocardial Infarction: Protection by Ischemic Pre- and Postconditioning'. *Herz* 33 (2), 88-100

Sprague, A. H. and Khalil, R. A. (2009) 'Inflammatory Cytokines in Vascular Dysfunction and Vascular Disease'. *Biochemical Pharmacology* 78 (6), 539-552

Stern, S., Behar, S., and Gottlieb, S. (2003) 'Aging and Diseases of the Heart'. *Circulation* 108 (14), e99-e101

Stowe, D. F. and Camara, A. K. (2009) 'Mitochondrial Reactive Oxygen Species Production in Excitable Cells: Modulators of Mitochondrial and Cell Function'. *Antioxidants & Redox Signaling* 11 (6), 1373-1414

Steelman, L. S., Chappell, W. H., Abrams, S. L., Kempf, R. C., Long, J., Laidler, P., Mijatovic, S., Maksimovic-Ivanic, D., Stivala, F., Mazzarino, M. C., Donia, M., Fagone, P., Malaponte, G., Nicoletti, F., Libra, M., Milella, M., Tafuri, A., Bonati, A., Basecke, J., Cocco, L., Evangelisti, C., Martelli, A. M., Montalto, G., Cervello, M., and McCubrey, J. A. (2011) 'Roles of the Raf/MEK/ERK and PI3K/PTEN/Akt/mTOR Pathways in Controlling Growth and Sensitivity to Therapy-Implications for Cancer and Aging'. *Aging* 3 (3), 192-222

Sui, T., Ma, L., Bai, X., Li, Q., and Xu, X. (2014) 'Resveratrol Inhibits the Phosphatidylinositide 3-kinase/protein Kinase B/mammalian Target of Rapamycin Signaling Pathway in the Human Chronic Myeloid Leukemia K562 Cell Line'. *Oncology Letters* 7 (6), 2093-2098

Sun, L. H., Li, H. Z., Han, L. P., Jiang, C. M., Zhao, Y. J., Gao, X. X., Tian, Y., and Xu, C. Q. (2007) 'Effect of Artemisinin on ischaemia/reperfusion Injury of Isolated Rat Myocardium'. *Zhongguo Zhong Yao Za Zhi = Zhongguo Zhongyao Zazhi = China Journal of Chinese Materia Medica* 32 (15), 1547-1551

Sun, Z., Tong, G., Ma, N., Li, J., Li, X., Li, S., Zhou, J., Xiong, L., Cao, F., Yao, L., Wang, H., and Shen, L. (2013) 'NDRG2: A Newly Identified Mediator of Insulin Cardioprotection Against Myocardial Ischaemia-Reperfusion Injury'. *Basic Research in Cardiology* 108 (3), 341-013-0341-5. Epub 2013 Mar 6

Swain, S. M., Whaley, F. S., and Ewer, M. S. (2003) 'Congestive Heart Failure in Patients Treated with Doxorubicin: A Retrospective Analysis of Three Trials'. *Cancer* 97 (11), 2869-2879

Swain, S. M. and Vici, P. (2004) 'The Current and Future Role of Dexrazoxane as a Cardioprotectant in Anthracycline Treatment: Expert Panel Review'. *Journal of Cancer Research and Clinical Oncology* 130 (1), 1-7

Tacar, O., Sriamornsak, P., and Dass, C. R. (2013) 'Doxorubicin: An Update on Anticancer Molecular Action, Toxicity and Novel Drug Delivery Systems'. *The Journal of Pharmacy and Pharmacology* 65 (2), 157-170

Takatani-Nakase, T. and Takahashi, K. (2010) 'Cardioprotective Activity of Urocortin by Preventing Caspase-Independent, Non-Apoptotic Death in Cultured Neonatal Rat Cardiomyocytes Exposed to Ischaemia'. *Biochemical and Biophysical Research Communications* 402 (2), 216-221

Takemura, G. and Fujiwara, H. (2007) 'Doxorubicin-Induced Cardiomyopathy from the Cardiotoxic Mechanisms to Management'. *Progress in Cardiovascular Diseases* 49 (5), 330-352

Tamareille, S., Mateus, V., Ghaboura, N., Jeanneteau, J., Croue, A., Henrion, D., Furber, A., and Prunier, F. (2011) 'RISK and SAFE Signaling Pathway Interactions in Remote Limb Ischemic Perconditioning in Combination with Local Ischemic Postconditioning'. *Basic Research in Cardiology* 106 (6), 1329-1339

Tang, Y., Zheng, J., Sun, Y., Wu, Z., Liu, Z., and Huang, G. (2009) 'MicroRNA-1 Regulates Cardiomyocyte Apoptosis by Targeting Bcl-2'. *International Heart Journal* 50 (3), 377-387

Tashakkor, A. Y., Moghaddamjou, A., Chen, L., and Cheung, W. Y. (2013) 'Predicting the Risk of Cardiovascular Comorbidities in Adult Cancer Survivors'. *Current Oncology (Toronto, Ont.)* 20 (5), e360-70

Tawfik, A. F., Bishop, S. J., Ayalp, A., and El-Feraly, F. S. (1990) 'Effects of Artemisinin, Dihydroartemisinin and Arteether on Immune Responses of Normal Mice'. *International Journal of Immunopharmacology* 12 (4), 385-389

Tebbi, C. K., London, W. B., Friedman, D., Villaluna, D., De Alarcon, P. A., Constine, L. S., Mendenhall, N. P., Sposto, R., Chauvenet, A., and Schwartz, C. L. (2007) 'Dexrazoxane-Associated Risk for Acute Myeloid leukemia/myelodysplastic Syndrome and Other Secondary Malignancies in Pediatric Hodgkin's Disease'. *Journal of Clinical Oncology : Official Journal of the American Society of Clinical Oncology* 25 (5), 493-500

Tebbi, C. K., London, W. B., Friedman, D., Villaluna, D., De Alarcon, P. A., Constine, L. S., Mendenhall, N. P., Sposto, R., Chauvenet, A., and Schwartz, C. L. (2007) 'Dexrazoxane-Associated Risk for Acute Myeloid leukemia/myelodysplastic Syndrome and Other Secondary Malignancies in Pediatric Hodgkin's Disease'. *Journal of Clinical Oncology : Official Journal of the American Society of Clinical Oncology* 25 (5), 493-500

Thomas, S., Quinn, B. A., Das, S. K., Dash, R., Emdad, L., Dasgupta, S., Wang, X. Y., Dent, P., Reed, J. C., Pellecchia, M., Sarkar, D., and Fisher, P. B. (2013) 'Targeting the Bcl-2 Family for Cancer Therapy'. *Expert Opinion on Therapeutic Targets* 17 (1), 61-75

Thompson, D. S., Naqvi, N., Juul, S. M., Swanton, R. H., Wilmshurst, P., Coltart, D. J., Jenkins, B. S., and Webb-Peploe, M. M. (1982) 'Cardiac Work and Myocardial Substrate Extraction in Congestive Cardiomyopathy'. *British Heart Journal* 47 (2), 130-136

Tin, A. S., Sundar, S. N., Tran, K. Q., Park, A. H., Poindexter, K. M., and Firestone, G. L. (2012) 'Antiproliferative Effects of Artemisinin on Human Breast Cancer Cells Requires the Downregulated Expression of the E2F1 Transcription Factor and Loss of E2F1-Target Cell Cycle Genes'. *Anti-Cancer Drugs* 23 (4), 370-379

Toldo, S., Goehe, R. W., Lotrionte, M., Mezzaroma, E., Sumner, E. T., Biondi-Zoccai, G. G., Seropian, I. M., Van Tassell, B. W., Loperfido, F., Palazzoni, G., Voelkel, N. F., Abbate, A., and Gewirtz, D. A. (2013) 'Comparative Cardiac Toxicity of Anthracyclines in Vitro and in Vivo in the Mouse'. *PloS One* 8 (3), e58421

Tomai, F., Crea, F., Chiariello, L., and Gioffrè, P. A. (1999) 'Ischemic Preconditioning in Humans: Models, Mediators, and Clinical Relevance'. *Circulation* 100 (5), 559-563

Tomai, F., Crea, F., Gaspardone, A., Versaci, F., Ghini, A. S., Ferri, C., Desideri, G., Chiariello, L., and Gioffre, P. A. (1999) 'Effects of Naloxone on Myocardial Ischemic Preconditioning in Humans'. *Journal of the American College of Cardiology* 33 (7), 1863-1869

Tomiyama, H., Nakayama, T., Watanabe, G., Shiojima, K., Sakuma, Y., Yamamoto, A., Imai, Y., Yoshida, H., and Doba, N. (1999) 'Effects of Short-Acting and Long-Acting Loop Diuretics on HR Variability in Patients with Chronic Compensated Congestive Heart Failure'. *American Heart Journal* 137 (3), 543-548

Tompkins, A. J., Burwell, L. S., Digerness, S. B., Zaragoza, C., Holman, W. L., and Brookes, P. S. (2006) 'Mitochondrial Dysfunction in Cardiac ischaemia–reperfusion Injury: ROS

from Complex I, without Inhibition'. *Biochimica Et Biophysica Acta (BBA) - Molecular Basis of Disease* 1762 (2), 223-231

Toller, W., Heringlake, M., Guarracino, F., Algotsson, L., Alvarez, J., Argyriadou, H., Ben-Gal, T., Černý, V., Cholley, B., Eremenko, A., Guerrero-Orriach, J. L., Järvelä, K., Karanovic, N., Kivikko, M., Lahtinen, P., Lomivorotov, V., Mehta, R. H., Mušič, Š., Pollesello, P., Rex, S., Riha, H., Rudiger, A., Salmenperä, M., Szudi, L., Tritapepe, L., Wyncoll, D., and Öwall, A. (2015) 'Preoperative and Perioperative use of Levosimendan in Cardiac Surgery: European Expert Opinion'. *International Journal of Cardiology* 184 (0), 323-336

Trump, B. F., Berezesky, I. K., Chang, S. H., and Phelps, P. C. (1997) 'The Pathways of Cell Death: Oncosis, Apoptosis, and Necrosis'. *Toxicologic Pathology* 25 (1), 82-88

Trochu, J. N., Bouhour, J. B., Kaley, G., and Hintze, T. H. (2000) 'Role of Endothelium-Derived Nitric Oxide in the Regulation of Cardiac Oxygen Metabolism: Implications in Health and Disease'. *Circulation Research* 87 (12), 1108-1117

Tsikas, D. (2007) 'Analysis of Nitrite and Nitrate in Biological Fluids by Assays Based on the Griess Reaction: Appraisal of the Griess Reaction in the l-arginine/nitric Oxide Area of Research'. *Journal of Chromatography B* 851 (1–2), 51-70

Tsuruta, F., Masuyama, N., and Gotoh, Y. (2002) 'The Phosphatidylinositol 3-Kinase (PI3K)-Akt Pathway Suppresses Bax Translocation to Mitochondria'. *Journal of Biological Chemistry* 277 (16), 14040-14047

Uhlemann, A. C. and Fidock, D. A. (2012) 'Loss of Malarial Susceptibility to Artemisinin in Thailand'. *Lancet* 379 (9830), 1928-1930

Uhlemann, A. C., Cameron, A., Eckstein-Ludwig, U., Fischbarg, J., Iserovich, P., Zuniga, F. A., East, M., Lee, A., Brady, L., Haynes, R. K., and Krishna, S. (2005) 'A Single Amino Acid Residue can Determine the Sensitivity of SERCAs to Artemisinins'. *Nature Structural & Molecular Biology* 12 (7), 628-629

van Agtmael, M. A., Eggelte, T. A., and van Boxtel, C. J. (1999) 'Artemisinin Drugs in the Treatment of Malaria: From Medicinal Herb to Registered Medication'. *Trends in Pharmacological Sciences* 20 (5), 199-205

van Dalen, E. C., Caron, H. N., Dickinson, H. O., and Kremer, L. C. (2011) 'Cardioprotective Interventions for Cancer Patients Receiving Anthracyclines'. *The van der Horst, I. C., Ligtenberg, J. J., Bilo, H. J., Zijlstra, F., and Gans, R. O. (2003) 'Glucose-Insulin-Potassium Infusion in Sepsis and Septic Shock: No Hard Evidence Yet'. Critical Care (London, England) 7 (1), 13-15 Cochrane Database of Systematic Reviews* (6):CD003917. doi (6), CD003917

van Hensbroek, M. B., Onyiorah, E., Jaffar, S., Schneider, G., Palmer, A., Frenkel, J., Enwere, G., Forck, S., Nusmeijer, A., Bennett, S., Greenwood, B., and Kwiatkowski, D. (1996) 'A Trial of Artemether Or Quinine in Children with Cerebral Malaria'. *N Engl J Med* 335 (2), 69-75

Van Noorden, C. J. (2001) 'The History of Z-VAD-FMK, a Tool for Understanding the Significance of Caspase Inhibition'. *Acta Histochemica* 103 (3), 241-251

van Rooij, E. (2011) 'The Art of MicroRNA Research'. *Circulation Research* 108 (2), 219-234

van Vugt, M., Ezzet, F., Nosten, F., Gathmann, I., Wilairatana, P., Looareesuwan, S., and White, N. J. (1999) 'No Evidence of Cardiotoxicity during Antimalarial Treatment with Artemether-Lumefantrine'. *The American Journal of Tropical Medicine and Hygiene* 61 (6), 964-967

van Wanrooij, E. J., de Vos, P., Bixel, M. G., Vestweber, D., van Berkel, T. J., and Kuiper, J. (2008) 'Vaccination Against CD99 Inhibits Atherogenesis in Low-Density Lipoprotein Receptor-Deficient Mice'. *Cardiovascular Research*78 (3), 590-596

Vanden Berghe, T., Vanlangenakker, N., Parthoens, E., Deckers, W., Devos, M., Festjens, N., Guerin, C. J., Brunk, U. T., Declercq, W., and Vandenabeele, P. (2010) 'Necroptosis,

Necrosis and Secondary Necrosis Converge on Similar Cellular Disintegration Features'. *Cell Death and Differentiation* 17 (6), 922-930

Vanden Berghe, T., Linkermann, A., Jouan-Lanhouet, S., Walczak, H., and Vandenabeele, P. (2014) 'Regulated Necrosis: The Expanding Network of Non-Apoptotic Cell Death Pathways'. *Nature Reviews.Molecular Cell Biology*15 (2), 135-147

Vasquez-Vivar, J., Martasek, P., Hogg, N., Masters, B. S., Pritchard, K. A.,Jr, and Kalyanaraman, B. (1997) 'Endothelial Nitric Oxide Synthase-Dependent Superoxide Generation from Adriamycin'. *Biochemistry* 36 (38), 11293-11297

Volkova, M. and Russell, R.,3rd (2011) 'Anthracycline Cardiotoxicity: Prevalence, Pathogenesis and Treatment'. *Current Cardiology Reviews* 7 (4), 214-220

Vejpongsa, P. and Yeh, E. T. (2014) 'Wrestling with Heart Failure: SUMO-1 to the Rescue'. *Circulation Research* 114 (10), 1561-1563

Venditti, P., Di Stefano, L., and Di Meo, S. (2013) 'Mitochondrial Metabolism of Reactive Oxygen Species'. *Mitochondrion* 13 (2), 71-82

Verhagen, A. M., Ekert, P. G., Pakusch, M., Silke, J., Connolly, L. M., Reid, G. E., Moritz, R. L., Simpson, R. J., and Vaux, D. L. (2000) 'Identification of DIABLO, a Mammalian Protein that Promotes Apoptosis by Binding to and Antagonizing IAP Proteins'. *Cell* 102 (1), 43-53

Verma, S., Fedak, P. W., Weisel, R. D., Butany, J., Rao, V., Maitland, A., Li, R. K., Dhillon, B., and Yau, T. M. (2002) 'Fundamentals of Reperfusion Injury for the Clinical Cardiologist'. *Circulation* 105 (20), 2332-2336

Vermes, I., Haanen, C., and Reutelingsperger, C. (2000) 'Flow Cytometry of Apoptotic Cell Death'. *Journal of Immunological Methods* 243 (1-2), 167-190

Vermeulen, K., Van Bockstaele, D. R., and Berneman, Z. N. (2005) 'Apoptosis: Mechanisms and Relevance in Cancer'. *Annals of Hematology* 84 (10), 627-639

Vogel, J. H. K., Bolling, S. F., Costello, R. B., Guarneri, E. M., Krucoff, M. W., Longhurst, J. C., Olshansky, B., Pelletier, K. R., Tracy, C. M., Vogel, R. A., Vogel, R. A., Abrams, J., Anderson, J. L., Bates, E. R., Brodie, B. R., Grines, C. L., Danias, P. G., Gregoratos, G., Hlatky, M. A., Hochman, J. S., Kaul, S., Lichtenberg, R. C., Lindner, J. R., O'Rourke, R. A., Pohost, G. M., Schofield, R. S., Shubrooks, S. J., Tracy, C. M., and Winters Jr, W. L. (2005) 'Integrating Complementary Medicine into Cardiovascular Medicine: A Report of the American College of Cardiology Foundation Task Force on Clinical Expert Consensus Documents (Writing Committee to Develop an Expert Consensus Document on Complementary and Integrative Medicine)'. *Journal of the American College of Cardiology* 46 (1), 184-221

Volkova, M. and Russell, R.,3rd (2011) 'Anthracycline Cardiotoxicity: Prevalence, Pathogenesis and Treatment'. *Current Cardiology Reviews* 7 (4), 214-220

Wahl, S. M., McCartney-Francis, N., Chan, J., Dionne, R., Ta, L., and Orenstein, J. M. (2003) 'Nitric Oxide in Experimental Joint Inflammation. Benefit Or Detriment?'. *Cells, Tissues, Organs* 174 (1-2), 26-33

Wallach, D., Kang, T. B., and Kovalenko, A. (2008) 'The Extrinsic Cell Death Pathway and the Elan Mortel'. *Cell Death and Differentiation* 15 (10), 1533-1541

Walsh, J. J., Coughlan, D., Heneghan, N., Gaynor, C., and Bell, A. (2007) 'A Novel Artemisinin-Quinine Hybrid with Potent Antimalarial Activity'. *Bioorganic & Medicinal Chemistry Letters* 17 (13), 3599-3602

Wang, B., Yang, H., Liu, Y. C., Jelinek, T., Zhang, L., Ruoslahti, E., and Fu, H. (1999) 'Isolation of High-Affinity Peptide Antagonists of 14-3-3 Proteins by Phage Display'. *Biochemistry* 38 (38), 12499-12504

Wang, J. and Yang, X. (2012) 'The Function of miRNA in Cardiac Hypertrophy'. *Cellular and Molecular Life Sciences : CMLS* 69 (21), 3561-3570

Wang, J., Wolin, M. S., and Hintze, T. H. (1993) 'Chronic Exercise Enhances Endothelium-Mediated Dilation of Epicardial Coronary Artery in Conscious Dogs.'. *Circulation Research* 73 (5), 829-838

Wang, J., Huang, L., Li, J., Fan, Q., Long, Y., Li, Y., and Zhou, B. (2010) 'Artemisinin Directly Targets Malarial Mitochondria through its Specific Mitochondrial Activation'. *PloS One* 5 (3), e9582

Wang, J., Tian, S., Petros, R. A., Napier, M. E., and Desimone, J. M. (2010) 'The Complex Role of Multivalency in Nanoparticles Targeting the Transferrin Receptor for Cancer Therapies'. *Journal of the American Chemical Society* 132 (32), 11306-11313

Wang, J. X., Hou, L. F., Yang, Y., Tang, W., Li, Y., and Zuo, J. P. (2009) 'SM905, an Artemisinin Derivative, Inhibited NO and Pro-Inflammatory Cytokine Production by Suppressing MAPK and NF-kappaB Pathways in RAW 264.7 Macrophages'. *Acta Pharmacologica Sinica* 30 (10), 1428-1435

Wang, R., Li, N., Zhang, Y., Ran, Y., and Pu, J. (2011) 'Circulating microRNAs are Promising Novel Biomarkers of Acute Myocardial Infarction'. *Internal Medicine (Tokyo, Japan)* 50 (17), 1789-1795

Wang, S. J., Sun, B., Cheng, Z. X., Zhou, H. X., Gao, Y., Kong, R., Chen, H., Jiang, H. C., Pan, S. H., Xue, D. B., and Bai, X. W. (2011) 'Dihydroartemisinin Inhibits Angiogenesis in Pancreatic Cancer by Targeting the NF-kappaB Pathway'. *Cancer Chemotherapy and Pharmacology* 68 (6), 1421-1430

Wang, J., Huang, L., Li, J., Fan, Q., Long, Y., Li, Y., and Zhou, B. (2010) 'Artemisinin Directly Targets Malarial Mitochondria through its Specific Mitochondrial Activation'. *PloS One* 8 (5), 3

Wang, X., McCullough, K. D., Franke, T. F., and Holbrook, N. J. (2000) 'Epidermal Growth Factor Receptor-Dependent Akt Activation by Oxidative Stress Enhances Cell Survival'. *Journal of Biological Chemistry* 275 (19), 14624-14631

Wang, Y., Cheng, X., Wang, P., Wang, L., Fan, J., Wang, X., and Liu, Q. (2014) 'Investigating Migration Inhibition and Apoptotic Effects of Fomitopsis Pinicola Chloroform Extract on Human Colorectal Cancer SW-480 Cells'. *PloS One* 9 (7), e101303

Wang, Y., John, R., Chen, J., Richardson, J. A., Shelton, J. M., Bennett, M., Zhou, X. J., Nagami, G. T., Zhang, Y., Wu, Q. Q., and Lu, C. Y. (2009) 'IRF-1 Promotes Inflammation Early After Ischemic Acute Kidney Injury'. *Journal of the American Society of Nephrology: JASN* 20 (7), 1544-1555

Wang, Y. Y., Liu, Y. X., Xie, Q. B., and Liu, G. (2012) 'Effects of Dihydroartemisinin on Collagen II-Induced Arthritis in Rats Model'. *Sichuan Da Xue Xue Bao.Yi Xue Ban = Journal of Sichuan University.Medical Science Edition*43 (6), 851-854

Wang, Z., Qiu, J., Guo, T. B., Liu, A., Wang, Y., Li, Y., and Zhang, J. Z. (2007) Anti-Inflammatory Properties and Regulatory Mechanism of a Novel Derivative of Artemisinin in Experimental Autoimmune Encephalomyelitis'.*Journal of Immunology* 1;179(9):5958-65

Wattanapitayakul, S. K., Chularojmontri, L., Herunsalee, A., Charuchongkolwongse, S., Niumsakul, S., and Bauer, J. A. (2005) 'Screening of Antioxidants from Medicinal Plants for Cardioprotective Effect Against Doxorubicin Toxicity'. *Basic & Clinical Pharmacology & Toxicology* 96 (1), 80-87

Webster, K. A. (2012) 'Mitochondrial Membrane Permeabilization and Cell Death during Myocardial Infarction: Roles of Calcium and Reactive Oxygen Species'. *Future Cardiology* 8 (6), 863-884

Weiss, J. B., Eisenhardt, S. U., Stark, G. B., Bode, C., Moser, M., and Grundmann, S. (2012) 'MicroRNAs in Ischaemia-Reperfusion Injury'. *American Journal of Cardiovascular Disease* 2 (3), 237-247

Werf, F. V. d., Ludbrook, P. A., Bergmann, S. R., Tiefenbrunn, A. J., Fox, K. A. A., Geest, H. d., Verstraete, M., Collen, D., and Sobel, B. E. (1984) 'Coronary Thrombolysis with

Tissue-Type Plasminogen Activator in Patients with Evolving Myocardial Infarction'. *New England Journal of Medicine* 310 (10), 609-613

Wesche, D. E., Lomas-Neira, J. L., Perl, M., Chung, C. S., and Ayala, A. (2005) 'Leukocyte Apoptosis and its Significance in Sepsis and Shock'. *Journal of Leukocyte Biology* 78 (2), 325-337

Wheeler-Jones, C. P. (2005) 'Cell Signalling in the Cardiovascular System: An Overview'. *Heart (British Cardiac Society)* 91 (10), 1366-1374

White, N. J. (2007) 'Cardiotoxicity of Antimalarial Drugs'. *The Lancet.Infectious Diseases* 7 (8), 549-558

Whittington, H. J., Harding, I., Stephenson, C. I., Bell, R., Hausenloy, D. J., Mocanu, M. M., and Yellon, D. M. (2013) 'Cardioprotection in the Aging, Diabetic Heart: The Loss of Protective Akt Signalling'. *Cardiovascular Research*99 (4), 694-704

Willoughby JA, S., Sundar, S. N., Cheung, M., Tin, A. S., Modiano, J., and Firestone, G. L. (2009) 'Artemisinin Blocks Prostate Cancer Growth and Cell Cycle Progression by Disrupting Sp1 Interactions with the Cyclin-Dependent Kinase-4 (CDK4) Promoter and Inhibiting CDK4 Gene Expression'. *The Journal of Biological Chemistry* 284 (4), 2203-2213

Willoughby, J. A. (2002) *Artemisinin Regulation of Proliferation and Disruption of Androgen Responsiveness of Human Prostrate Cancer Cells*. [online] Ph.D in molecular and cell biology thesis or dissertation. Berkeley: University of California

Wink, D. A., Kasprzak, K. S., Maragos, C. M., Elespuru, R. K., Misra, M., Dunams, T. M., Cebula, T. A., Koch, W. H., Andrews, A. W., and Allen, J. S. (1991) 'DNA Deaminating Ability and Genotoxicity of Nitric Oxide and its Progenitors'. *Science (New York, N.Y.)* 254 (5034), 1001-1003

Wright, G., Kingston, M. A., and Ross, I. S. (1995) 'Role of Metabolic Acidosis on Cardiac Mechanical Performance during Severe Acute Hypoxia and Reoxygenation is Small and Transient'. *Cardiovascular Research* 29 (5), 611-615

Writing Group Members, Roger, V. L., Go, A. S., Lloyd-Jones, D. M., Benjamin, E. J., Berry, J. D., Borden, W. B., Bravata, D. M., Dai, S., Ford, E. S., Fox, C. S., Fullerton, H. J., Gillespie, C., Hailpern, S. M., Heit, J. A., Howard, V. J., Kissela, B. M., Kittner, S. J., Lackland, D. T., Lichtman, J. H., Lisabeth, L. D., Makuc, D. M., Marcus, G. M., Marelli, A., Matchar, D. B., Moy, C. S., Mozaffarian, D., Mussolino, M. E., Nichol, G., Paynter, N. P., Soliman, E. Z., Sorlie, P. D., Sotoodehnia, N., Turan, T. N., Virani, S. S., Wong, N. D., Woo, D., and Turner, M. B. (2012) 'Heart Disease and Stroke Statistics—2012 Update: A Report from the American Heart Association'. *Circulation* 125 (1), e2-e220

Wu, B., Hu, K., Li, S., Zhu, J., Gu, L., Shen, H., Hambly, B. D., Bao, S., and Di, W. (2012) 'Dihydroartiminisin Inhibits the Growth and Metastasis of Epithelial Ovarian Cancer'. *Oncology Reports* 27 (1), 101-108

Wu, C. D., Kuo, Y. S., Wu, H. C., and Lin, C. T. (2011) 'MicroRNA-1 Induces Apoptosis by Targeting Prothymosin Alpha in Nasopharyngeal Carcinoma Cells'. *Journal of Biomedical Science* 18, 80-0127-18-80

Wu, H., Che, X., Zheng, Q., Wu, A., Pan, K., Shao, A., Wu, Q., Zhang, J., and Hong, Y. (2014) 'Caspases: A Molecular Switch Node in the Crosstalk between Autophagy and Apoptosis'. *International Journal of Biological Sciences* 10 (9), 1072-1083

Wu, S. N., Nakajima, T., Yamashita, T., Hamada, E., Hazama, H., Iwasawa, K., Omata, M., and Kurachi, Y. (1994) 'Molecular Mechanism of Cibenzoline-Induced Anticholinergic Action in Single Atrial Myocytes: Comparison with Effect of Disopyramide'. *Journal of Cardiovascular Pharmacology* 23 (4), 618-623

Wu, W., Lee, W. L., Wu, Y. Y., Chen, D., Liu, T. J., Jang, A., Sharma, P. M., and Wang, P. H. (2000) 'Expression of Constitutively Active Phosphatidylinositol 3-Kinase Inhibits Activation of Caspase 3 and Apoptosis of Cardiac Muscle Cells'. *The Journal of Biological Chemistry* 275 (51), 40113-40119

Xia, M., Huang, R., Witt, K. L., Southall, N., Fostel, J., Cho, M. H., Jadhav, A., Smith, C. S., Inglese, J., Portier, C. J., Tice, R. R., and Austin, C. P. (2008) 'Compound Cytotoxicity Profiling using Quantitative High-Throughput Screening'. *Environmental Health Perspectives* 116 (3), 284-291

Xia Ma, F. and Han, Z. C. (2005) 'Statins, Nitric Oxide and Neovascularization'. *Cardiovascular Drug Reviews* 23 (4), 281-292

Xiao, J. and Chen, Y. H. (2010) 'MicroRNAs: Novel Regulators of the Heart'. *Journal of Thoracic Disease* 2 (1), 43-47

Xie, L. H., Li, Q., Zhang, J., and Weina, P. J. (2009) 'Pharmacokinetics, Tissue Distribution and Mass Balance of Radiolabeled Dihydroartemisinin in Male Rats'. *Malaria Journal* 8, 112-2875-8-112

Xiong, Z., Sun, G., Zhu, C., Cheng, B., Zhang, C., Ma, Y., and Dong, Y. (2010) 'Artemisinin, an Anti-Malarial Agent, Inhibits Rat Cardiac Hypertrophy Via Inhibition of NF-kappaB Signaling'. *European Journal of Pharmacology*649 (1-3), 277-284

Xu, C., Lu, Y., Pan, Z., Chu, W., Luo, X., Lin, H., Xiao, J., Shan, H., Wang, Z., and Yang, B. (2007) 'The Muscle-Specific microRNAs miR-1 and miR-133 Produce Opposing Effects on Apoptosis by Targeting HSP60, HSP70 and Caspase-9 in Cardiomyocytes'. *Journal of Cell Science* 120 (Pt 17), 3045-3052

Xu, H., He, Y., Yang, X., Liang, L., Zhan, Z., Ye, Y., Yang, X., Lian, F., and Sun, L. (2007) Anti-Malarial Agent Artesunate Inhibits TNF-α-Induced Production of Proinflammatory Cytokines Via Inhibition of NF-κB and PI3 kinase/Akt Signal Pathway in Human Rheumatoid Arthritis Fibroblast-Like Synoviocytes.

Xu, Z., Alloush, J., Beck, E., and Weisleder, N. (2014) 'A Murine Model of Myocardial Ischaemia-Reperfusion Injury through Ligation of the Left Anterior Descending Artery'. *Journal of Visualized Experiments : JoVE* (86). doi (86), 10.3791/51329

Yaghi, A., Mehta, S., and McCormack, D. G. (2002) 'Delayed Rectifier Potassium Channels Contribute to the Depressed Pulmonary Artery Contractility in Pneumonia'. *Journal of Applied Physiology (Bethesda, Md.: 1985)* 93 (3), 957-965

Yamashiro, S., Noguchi, K., Matsuzaki, T., Miyagi, K., Nakasone, J., Sakanashi, M., Sakanashi, M., Kukita, I., Aniya, Y., and Sakanashi, M. (2003) 'Cardioprotective Effects of Extracts from Psidium Guajava L and Limonium Wrightii, Okinawan Medicinal Plants, Against Ischaemia-Reperfusion Injury in Perfused Rat Hearts'. *Pharmacology* 67 (3), 128-135

Yang, B., Lin, H., Xiao, J., Lu, Y., Luo, X., Li, B., Zhang, Y., Xu, C., Bai, Y., Wang, H., Chen, G., and Wang, Z. (2007) 'The Muscle-Specific microRNA miR-1 Regulates Cardiac Arrhythmogenic Potential by Targeting GJA1 and KCNJ2'. *Nature Medicine* 13 (4), 486-491

Yang, B. F., Lu, Y. J., and Wang, Z. G. (2009) 'MicroRNAs and Apoptosis: Implications in the Molecular Therapy of Human Disease'. *Clinical and Experimental Pharmacology & Physiology* 36 (10), 951-960

Yang, B. F., Luo, D. L., Bao, L. H., Zhang, Y. C., and Wang, H. Z. (1998) 'Artemisinin Blocks Activating and Slowly Activating K+ Current in Guinea Pig Ventricular Myocytes'. *Zhongguo Yao Li Xue Bao = Acta Pharmacologica Sinica* 19 (3), 269-272

Yang, Z., Ding, J., Yang, C., Gao, Y., Li, X., Chen, X., Peng, Y., Fang, J., and Xiao, S. (2012) 'Immunomodulatory and Anti-Inflammatory Properties of Artesunate in Experimental Colitis'. *Current Medicinal Chemistry* 19 (26), 4541-4551

Yao, R. and Cooper, G. (1995) 'Requirement for Phosphatidylinositol-3 Kinase in the Prevention of Apoptosis by Nerve Growth Factor'. *Science* 267 (5206), 2003-2006

Yao, H., Han, X., and Han, X. (2014) 'The Cardioprotection of the Insulin-Mediated PI3K/Akt/mTOR Signaling Pathway'. *American Journal of Cardiovascular Drugs : Drugs, Devices, and Other Interventions*

Yatuv, R., Robinson, M., Dayan-Tarshish, I., and Baru, M. (2010) 'The use of PEGylated Liposomes in the Development of Drug Delivery Applications for the Treatment of Hemophilia'. *International Journal of Nanomedicine* 5, 581-591

Ye, Y., Perez-Polo, J. R., and Birnbaum, Y. (2010) 'Protecting Against Ischaemia-Reperfusion Injury: Antiplatelet Drugs, Statins, and their Potential Interactions'. *Annals of the New York Academy of Sciences* 1207, 76-82

Yeh, E. T. H., Tong, A. T., Lenihan, D. J., Yusuf, S. W., Swafford, J., Champion, C., Durand, J., Gibbs, H., Zafarmand, A. A., and Ewer, M. S. (2004) 'Cardiovascular Complications of Cancer Therapy: Diagnosis, Pathogenesis, and Management'. *Circulation* 109 (25), 3122-3131

Yellon, D. M., Alkhulaifi, A. M., and Pugsley, W. B. (1993) 'Preconditioning the Human Myocardium'. *Lancet (London, England)* 342 (8866), 276-277

Yellon, D. M. and Hausenloy, D. J. (2007) 'Myocardial Reperfusion Injury'. *The New England Journal of Medicine* 357 (11), 1121-1135

Yellon, D. M. and Dana, A. (2000) 'The Preconditioning Phenomenon: A Tool for the Scientist Or a Clinical Reality?'. *Circulation Research* 87 (7), 543-550

Yellon, D. M. and Hausenloy, D. J. (2007) 'Myocardial Reperfusion Injury'. *N Engl J Med* 357 (11), 1121-1135

Yin, J. Y., Wang, H. M., Wang, Q. J., Dong, Y. S., Han, G., Guan, Y. B., Zhao, K. Y., Qu, W. S., Yuan, Y., Gao, X. X., Jing, S. F., and Ding, R. G. (2014) 'Subchronic Toxicological Study of Two Artemisinin Derivatives in Dogs'. *PloS One* 9 (4), e94034

Yoon, M. K., Mitrea, D. M., Ou, L., and Kriwacki, R. W. (2012) 'Cell Cycle Regulation by the Intrinsically Disordered Proteins p21 and p27'. *Biochemical Society Transactions* 40 (5), 981-988

Young, I. S. and Woodside, J. V. (2001) 'Antioxidants in Health and Disease'. *Journal of Clinical Pathology* 54 (3), 176-186

Yuan, S., Yu, X., Asara, J. M., Heuser, J. E., Ludtke, S. J., and Akey, C. W. (2011) 'The Holo-Apoptosome: Activation of Procaspase-9 and Interactions with Caspase-3'. *Structure (London, England : 1993)* 19 (8), 1084-1096

Zaki, S. A., Shanbag, P., Lad, V., and Shenoy, P. (2011) 'Sodium Artesunate-Induced Diuresis in a Patient with Malaria'. *Indian Journal of Pharmacology* 43 (4), 472-473

Zen, K. and Zhang, C. (2012) 'Circulating MicroRNAs: A Novel Class of Biomarkers to Diagnose and Monitor Human Cancers'. *Medicinal Research Reviews* 32 (2), 326-348

Zeng, Q. P., Xiao, N., Wu, P., Yang, X. Q., Zeng, L. X., Guo, X. X., Zhang, P. Z., and Qiu, F. (2011) 'Artesunate Potentiates Antibiotics by Inactivating Heme-Harbouring Bacterial Nitric Oxide Synthase and Catalase'. *BMC Research Notes* 4, 223

Zhang, H., Li, M., Han, Y., Hong, L., Gong, T., Sun, L., and Zheng, X. (2010) 'Down-Regulation of miR-27a might Reverse Multidrug Resistance of Esophageal Squamous Cell Carcinoma'. *Digestive Diseases and Sciences* 55 (9), 2545-2551

Zhao, H., Shen, J., Medico, L., Wang, D., Ambrosone, C. B., and Liu, S. (2010) 'A Pilot Study of Circulating miRNAs as Potential Biomarkers of Early Stage Breast Cancer'. *PloS One* 5 (10), e13735

Zhang, S., Liu, X., Bawa-Khalfe, T., Lu, L. S., Lyu, Y. L., Liu, L. F., and Yeh, E. T. (2012) 'Identification of the Molecular Basis of Doxorubicin-Induced Cardiotoxicity'. *Nature Medicine* 18 (11), 1639-1642

Zhang, Y. and Huo, Y. (2011) 'Early Reperfusion Strategy for Acute Myocardial Infarction: A Need for Clinical Implementation'. *Journal of Zhejiang University.Science.B* 12 (8), 629-632

Zhao, D. Y., Zhao, M. Q., and Wu, W. K. (2008) 'Study on Activity and Mechanism of Sini Decoction Anti-Mitochondrial Oxidation Injury Caused by Myocardial ischemia/reperfusion'. *Zhong Yao Cai = Zhongyaocai = Journal of Chinese Medicinal Materials* 31 (11), 1681-1685

Zhang, Y., Tocchetti, C. G., Krieg, T., and Moens, A. L. (2012) 'Oxidative and Nitrosative Stress in the Maintenance of Myocardial Function'. *Free Radical Biology & Medicine* 53 (8), 1531-1540

Zhang, P., Lu, Y., Yu, D., Zhang, D., and Hu, W. (2015) 'TRAP1 Provides Protection Against Myocardial Ischemia-Reperfusion Injury by Ameliorating Mitochondrial Dysfunction'. *Cellular Physiology and Biochemistry: International Journal of Experimental Cellular Physiology, Biochemistry, and Pharmacology* 36 (5), 2072-2082

Zhao, G., Shen, W., Xu, X., Ochoa, M., Bernstein, R., and Hintze, T. H. (1995) 'Selective Impairment of Vagally Mediated, Nitric Oxide–Dependent Coronary Vasodilation in Conscious Dogs After Pacing-Induced Heart Failure'. *Circulation* 91 (10), 2655-2663

Zhao, X., Yang, L., and Hu, J. (2011) 'Down-Regulation of miR-27a might Inhibit Proliferation and Drug Resistance of Gastric Cancer Cells'. *Journal of Experimental & Clinical Cancer Research: CR* 30, 55-9966-30-55

Zhao, Z. Q. and Vinten-Johansen, J. (2002) 'Myocardial Apoptosis and Ischemic Preconditioning'. *Cardiovascular Research* 55 (3), 438-455

Zheng, W. H. and Quirion, R. (2006) 'Insulin-Like Growth Factor-1 (IGF-1) Induces the activation/phosphorylation of Akt Kinase and cAMP Response Element-Binding Protein (CREB) by Activating Different Signaling Pathways in PC12 Cells'. *BMC Neuroscience* 7, 51

Zhou, H. J., Wang, Z., and Li, A. (2008) 'Dihydroartemisinin Induces Apoptosis in Human Leukemia Cells HL60 Via Downregulation of Transferrin Receptor Expression'. *Anti-Cancer Drugs* 19 (3), 247-255

Zhou, S., Starkov, A., Froberg, M. K., Leino, R. L., and Wallace, K. B. (2001) 'Cumulative and Irreversible Cardiac Mitochondrial Dysfunction Induced by Doxorubicin'. *Cancer Research* 61 (2), 771-777

Zhou, S., Sun, W., Zhang, Z., and Zheng, Y. (2014) 'The Role of Nrf2-Mediated Pathway in Cardiac Remodeling and Heart Failure'. *Oxidative Medicine and Cellular Longevity* 2014, 260429

Zhu, D. Y., Huang, B. S., Chen, Z. L., Yin, M. L., Yang, Y. M., Dai, M. L., Wang, B. D., and Huang, Z. H. (1983) 'Isolation and Identification of the Metabolite of Artemisinine in Human'. *Acta Pharmacologica Sinica* 4 (3), 194-197

Zoghbi, G. J., Dorfman, T. A., and Iskandrian, A. E. (2008) 'The Effects of Medications on Myocardial Perfusion'. *Journal of the American College of Cardiology* 52 (6), 401-416

Zweier, J. L. and Talukder, M. A. (2006) 'The Role of Oxidants and Free Radicals in Reperfusion Injury'. *Cardiovascular Research* 70 (2), 181-190

Published abstracts

Title: “The effect of low and high pO2 levels in a young and aged papillary muscle work-loop model subjected to simulated ischaemia-reperfusion”. S. Pollard, M. Babba, A. Hussain, R. James, H. Maddock (FCVB Conference London, 2012)

Title: “An investigation into the effect of Artemisinin on Myocardial Ischaemia reperfusion injury”. Babba M.A, Janneh O, Maddock H.L, and Hussain A. (Coventry University Research Symposium, 2012)

Title: "Artemisinin Protects The Myocardium From Ischaemia Reperfusion Injury Via PI3K/AKT/iNOS Cell Survival Pathway". Babba M.A, Janneh O., Maddock H.L, and Hussain A. (BPS Cell Signalling, Leicester 2014)

Title: “The anti-cancer drug Artemisinin protects against myocardial ischaemia-reperfusion injury via the recruitment of PI3K-AKT-p70S6k cell survival pathway”. Babba M.A, Janneh O, Maddock H.L, and Hussain A. (FCVB, Barcelona 2014)

Manuscripts to be submitted

Title: “Artemisinin Protects The Myocardium From Ischaemia Reperfusion Injury Via PI3K/AKT/iNOS Cell Survival Pathway” (Submitted for review)

Title: “Artemisinin alleviates doxorubicin induced cardiotoxicity in isolated rat hearts and cardiomyocytes via suppression of oxidative stress, inflammation and apoptosis” (Manuscript in preparation)

Title: “The ubiquitous role of nitric oxide in cancer and in cardioprotection” (Manuscript in preparation)